Micropropagation of *Guarianthe skinneri* (Bateman) Dressler et W. E. Higgins in Temporary Immersion Systems

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Abstract

This study's aim was to establish a protocol for the micropropagation of *G. skinneri* using temporary immersion system (TIS). Different concentrations of 6-Benzylaminopurine (0, 1, 2, and 3 mg L⁻¹), three different systems of cultivate semi-solid (SS) and liquid media under partial (PI) and temporary immersion systems (TIS), different compositions of the inorganic salts, and the number of subcultures were evaluated. The results showed a maximum of 16.56 shoots per explant obtained through TIS, adjusting all the parameters evaluated in our study. One higher number of shoots per explant was observed in the micropropagation of *G. skinneri* TIS compared to SS and PI. While the use of 3 mg L⁻¹ of BAP + MS (Murashige and Skoog) media was better than 3 mg L⁻¹ of BAP VW (Vacint and Went) for the generation of a greater number of shoots per explant, 6.33 and 2.72, respectively. The immersion frequency of 2 min every 4 h allowed the production to be scaled to 8.54 shoots per explant. While it was necessary to perform three subcultures every 30 days, to obtain 16.56 shoots per explant, a rooting phase was not required due to the generation of adventitious roots during the different subcultures. However, a phase of elongation of the regenerated plants with ½ MS + GA3 (gibberellic acid) was needed to guarantee 100% survival in the process of acclimatization. In conclusion, this plant production system can be applied for the commercial micropropagation of this species for ornamental purposes, as well as for its reintroduction in protected natural areas.

Keywords Micropropagation · Orchid · Ornamental · Temporary immersion bioreactor (TIB[®]) · Threatened species

Introduction

Guarianthe skinneri (Bateman) Dressler & W. E. Higgins (Orchidaceae), commonly known as cattleya Candelaria, is naturally distributed from Mexico to Panama (Bertolini et al. 2016; Damon et al. 2019). Currently, wild populations of this species have been reduced by illegal collection and fragmentation of its natural habitat (Damon 2013;

Coutiño-Cortés et al. 2018). In Mexico, the ornamental use of this species has led to its overexploitation, so it is currently listed as threatened by the Mexican regulations NOM-059-ECOL-2010 (SEMARNAT 2010). In addition, the natural reproduction of orchids from seed is slow, fragile, and requires strict illumination, temperature and humidity conditions (Bhattacharyya et al. 2018). Furthermore, the asexual propagation of orchids has time- and cost-related limitations that limit the production of vegetative shoots (Herrera et al. 2017; Yeung et al. 2018). Therefore, conservation and propagation strategies should be implemented for this important species aiming for the recovery of the populations currently threatened (Irawati 2013). In vitro propagation using tissue culture techniques is a feasible option to address this issue (Chugh et al. 2009; Irawati 2013).

The micropropagation of *G. skinneri* has already been described (Coello et al. 2010; Coutiño-Cortés et al. 2017). However, these protocols reported the use of the semisolid medium. Temporary immersion systems (TIS) are an alternative that combines the positive effects of liquid media

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that increase the multiplication coefficient with lower costs during the micropropagation of plants (Teisson and Alvard 1995; Escalona et al. 1999; Etienne and Berthouly 2002).

Various TIS designs have been reported; however, temporary immersion bioreactors (TIB®) or twin-jar bioreactors show advantages relative to other systems due to the broad availability of components, different capacity, and easy assembly (Aragón et al. 2014; Georgiev et al. 2014). Most micropropagation studies of orchids (Orchidaceae) using temporary immersion have involved the use of TIB® (Murthy et al. 2018; Shen and Hsu 2018; Zhang et al. 2018). However, according to Murthy et al. (2018), the nutritional requirements and particular parameters of TIS micropropagation should be tailored to each species studied. The objective of this study was to develop a system for the production of *G. skinneri* plants using TIB® aiming to contribute to the recovery of this important species.

Materials and methods

Plant material

G. skinneri (Bateman) Dressler & W. E. Higgins plantlets previously grown in vitro were used. These plantlets came from the asymbiotic germination of seeds grown in MS medium (Murashige and Skoog 1962, salts and vitamins) supplemented with 30 g L⁻¹ sucrose (w/v) and with no plant growth regulators (PGR). The medium pH was adjusted to 5.8, with 0.1 N NaOH and 0.1 N HCl and solidified with 2.2 g L⁻¹ Phytigel®, and autoclaved at 115 kPa for 15 min. Cultures were incubated at 25 ± 2 °C under illumination of 40–50 μmol m⁻² s⁻¹ using fluorescent lamps and a 16/8 h (light/dark) photoperiod.

Influence of 6-Benzylaminopurine (BAP) on in vitro regeneration in semisolid medium

Individual shoots measuring ~ 1 ± 0.2 cm in length were transferred to MS culture medium supplemented with 30 g L⁻¹ sucrose and 2.2 g L⁻¹ Phytigel® as gelling agent. We evaluated the effect of different concentrations of 6-Benzylaminopurine (BAP: 0, 1, 2, and 3 mg L⁻¹). The pH, sterilization and incubation conditions were the same as those described above. After 4 weeks of culture, the number of shoots per explant, shoot length, and the number of leaves were recorded.

Effect of the culture system on in vitro regeneration

Three different culture systems semi-solid (SS), partial immersion (PI), and temporary immersion system (TIS) (Fig. 1) were evaluated. SS is solid culture medium, PI

Fig. 1 Different culture systems used in the micropropagation of *G. skinneri*. **a** Semi-solid system (SS); **b** partial immersion system (PI); **c** temporary immersion system (TIS)

is liquid culture medium with partial immersion (~ 5 mm of the shoot base was submerged in liquid medium), and TIS is the temporary immersion bioreactors (TIB®). For SS and PI, jars of 500 mL each were used, 10 jars per treatment with 5 explants (100 mL of headspace volume for each explant). While for TIS, twin-jars of 1000 mL were used, 5 twin-jars per treatment with 10 explants each (100 mL of headspace volume for each explant). Individual shoots measuring 1 ± 0.2 cm in length were grown in three different culture systems. All culture systems evaluated used MS medium supplemented with 3 mg L⁻¹ BAP, 30 g L⁻¹ sucrose (liquid medium for PI and TIS and medium supplemented with 2.2 g L⁻¹ Phytigel® for SS). The volume of culture medium used for the SS and IP systems was 100 mL (20 mL for explant); for TIB® it was 200 mL (20 mL for explant).

The pH, sterilization and incubation conditions were the same as those described above. For TIS, the immersion frequency was 2 min every 4 h (2 min/4 h). After 4 weeks of

culture, the number of shoots per explant, shoot length, and the number of leaves were recorded.

Influence of the culture medium on the micropropagation of *G. skinneri* in TIS

Two different culture media, i.e., MS and VW (Vacint and Went 1949, supplemented with 0.4 mg L⁻¹ thiamine HCl, and 100 mg L⁻¹ myo-inositol), were evaluated in TIS. These were supplemented with 3 mg L⁻¹ 6-Benzylaminopurine (BAP) and 30 g L⁻¹ sucrose. TIS were used under the same conditions as in the previous experiment. The pH, sterilization and incubation conditions were the same as those described above. The immersion frequency was 2 min/4 h. After 4 weeks of culture, the number of shoots per explant, shoot length, and the number of leaves were recorded.

Effect of immersion frequency on the micropropagation of *G. skinneri* in TIS

Different immersion frequencies were assessed: 4, 6, and 8 h, with 2 min per immersion. All treatments used MS medium supplemented with 3 mg L⁻¹ BAP and 30 g L⁻¹ sucrose. The pH, sterilization and incubation conditions were the same as those described above. After 4 weeks of culture, the number of shoots per explant, shoot length, and number of leaves.

Influence of the number of subculture passes on in vitro development in TIS

Three subcultures were evaluated every 30 days (each subculture consisted in the disposal and renewal of the culture medium by the fresh medium in the same culture flask). All treatments used MS medium supplemented with 3 mg L⁻¹ BAP, and used an immersion frequency of 2 min/4 h. The pH, sterilization and incubation conditions were the same as those described above. After 4 weeks of culture, the number of shoots per explant, shoot length, and the number of leaves were recorded. Root formation was observed during the subculture process (it is not necessary to perform a rooting stage or add any PGR). Therefore, the number and length of roots per explant was evaluated.

Elongation of *G. skinneri* explants in TIS

Individual plants (rooted plants that came from the 3rd subculture, measuring 1.5 cm in length) were transferred to different concentrations of MS medium (50 and 100%), supplemented with 1.0 mg L⁻¹ gibberellic acid (GA₃) and 30 g L⁻¹ sucrose. 200 ml of culture medium were dosed to each TIB®. The pH, sterilization and incubation conditions were the same as those described above. The immersion

frequency was 2 min/4 h. After 4 weeks of culture, shoot length and number of leaves was recorded.

Acclimatization

Plantlets measuring 3–4 cm in length and showing optimal root development were used. These were rinsed with tap water; subsequently, shoots were planted in a sterile substrate, peat moss and agrolite (Tlalnepantla de Baz, Mexico) 1:1 v/v using individual pots. Plantlets were kept in 50% shade at 28–32 °C and 80–95% Relative Humidity (RH) under greenhouse conditions. Plantlets were irrigated with tap water three times per week; the leaf fertilizer Nitrofoska® N:P:K (25-10-17) was applied once a week. After eight weeks of cultivation, the percentage of survival was recorded.

Experimental design and statistical analysis

All experiments involved a fully random experimental design and were kept under identical conditions. Experiments were run in triplicate. All experiments included 50 explants per treatment. The data obtained were processed statistically with the software IBM SPSS Statistics (version 21). A Student's *t* test ($p \leq 0.05$) was used to compare the different culture media (MS and VW). While for the other experiments, an analysis of variance (ANOVA) was performed followed by a Tukey's test ($p \leq 0.05$).

Results

Influence of BAP on in vitro regeneration in semisolid medium

Significant differences were observed among the different BAP concentrations evaluated on the in vitro regeneration of shoots (Fig. 2a). The largest values of shoot number and shoot length occurred in culture medium supplemented with 3 mg L⁻¹ BAP, with 3.00 ± 0.13 shoots/explant measuring 1.05 ± 0.08 cm in length. Followed by 2.33 ± 0.13 shoots/explant and 1.20 ± 0.09 cm in 2 mg L⁻¹ of BAP, and 2.18 ± 0.20 shoots/explant and 0.96 ± 0.10 cm in 1 mg L⁻¹ of BAP. In contrast, the lowest number of shoots was recorded in the control treatment, with 1.25 ± 0.11 shoots per explant and measuring 0.81 ± 0.05 cm in length. The number of leaves obtained (4.06 ± 0.30) with 2 mg L⁻¹ BAP is higher than in all the other treatments.

Effect of the culture system on in vitro regeneration

About the different culture systems (Fig. 2b), the higher number of shoots was observed in TIS, with 6.06 ± 0.17

Fig. 2 Evaluation of different culture conditions in the micropropagation of *G. skinneri*. **a** Effect of different BAP concentrations in semi-solid medium; **b** effect of different culture systems on micropropagation (SS, PI, and TIS); **c** effect of the composition of the culture

shoots per explant, followed by the culture system in semi-solid medium (SS), with 2.96 ± 0.18 . The lowest number of shoots was obtained under partial immersion (PI), with 1.70 ± 0.19 shoots per explant (Fig. 3). With regard to shoot length, no significant differences were observed between culture systems. However, regarding the number of leaves, the greatest response was obtained in culture systems involving liquid medium (PI and TIS), with 2.58 ± 0.12 and

medium in TIS; **d** effect of different immersion frequencies in TIS. Bars indicate mean $\pm$ SE. Different letters (with the same font) indicate statistical significance (Tukey, $p \leq 0.05$) between columns

2.26 ± 0.05 , respectively, followed by the semi-solid medium system, with 1.90 ± 0.06 leaves.

Influence of culture medium on the micropropagation of *G. skinneri* in TIS

The assessment of the effect of the composition of the culture medium, shows a larger number of shoots per explant

Fig. 3 Micropropagation of *G. skinneri* in different in vitro culture systems. **a** 3 mg L⁻¹ BAP in semi-solid system; **b** 3 mg L⁻¹ BAP in partial Immersion system; **c** 3 mg L⁻¹ BAP in temporary immersion system

in MS medium, with 6.33 ± 0.24 shoots per explant, compared with the VW medium, with only 2.72 ± 0.20 shoots per explant. No significant differences were observed in shoot length and number of leaves (Fig. 2c).

Effect of immersion frequency on the micropropagation of *G. skinneri* in TIS

A larger number of shoots per explant was observed at 4 h immersion frequencies, with 8.54 ± 0.21 shoots per explant, followed by immersion every 6 h and 8 h, with 3.74 ± 0.20 and 3.72 ± 0.22 shoots per explant, respectively (Fig. 2d). No significant differences were observed in either shoot length or the number of leaves per explant (Fig. 4).

Influence of the number of subculture passes on in vitro development in TIS

Significant differences were observed in the number of shoots among the different subcultures. In general, shoot development increased in parallel with the number of subcultures passes (Table 1). The greatest development of explants was observed in the third subculture (90 days), which produced higher values in all variables evaluated. The presence of roots should be highlighted during the evaluation of subcultures (Fig. 5a).

Elongation of explants in TIS and acclimatization

As regards shoot elongation, longer shoots (3.67 ± 0.19 cm in length) were observed using $\frac{1}{2}$ MS medium supplemented with 1 mg L⁻¹ GA₃, followed by MS + 1 mg L⁻¹ GA₃, and MS with no growth regulator (1.95 ± 0.216 cm

Fig. 4 Effect of different immersion frequencies on micropropagation of *G. skinneri* in BIT®. **a** MS supplement with 3 mg L⁻¹ BAP, frequency immersion 2 min every 4 h; **b** MS supplement with 3 mg L⁻¹

BAP, frequency immersion 2 min every 8 h and **c** MS supplement with 3 mg L⁻¹ BAP, frequency immersion 2 min every 12 h

Table 1 Effect of subcultures in the micropropagation of *G. skinneri* using TIS

Multiplication cycles	No of shoots	Shoot length (cm)	No of leaves	No of roots	Root length (cm)
1st subculture	6.44 ± 0.20 ^c	1.19 ± 0.17 ^b	2.92 ± 0.12 ^b	1.81 ± 0.24 ^b	0.37 ± 0.19 ^c
2nd subculture	8.22 ± 0.44 ^b	1.34 ± 0.14 ^b	2.62 ± 0.18 ^b	6.60 ± 0.91 ^a	0.69 ± 0.16 ^b
3rd subculture	16.56 ± 0.86 ^a	1.61 ± 0.11 ^a	3.64 ± 0.11 ^a	6.32 ± 0.16 ^a	0.94 ± 0.17 ^a

Numbers represent mean ± SE (standard error). Means with different letters are significantly different (Tukey, $p \leq 0.05$)

Fig. 5 *G. skinneri* plants micropropagated in TIS. **a** Rooted plants after the 3rd subculture; **b** plants after 28 weeks in the acclimatization process**Table 2** Elongation of shoots of *G. skinneri* in TIS

Treatments	Shoot length (cm)	No of leaves
MS	1.19 ± 0.17 ^b	2.92 ± 0.12 ^b
MS + GA ₃	1.95 ± 0.16 ^b	2.25 ± 0.10 ^b
½ MS + GA ₃	3.67 ± 0.19 ^a	3.76 ± 0.10 ^a

Numbers represent mean ± SE (standard error). Means with different letters are significantly different (Tukey, $p \leq 0.05$)

MS medium Murashige and Skoog (1962), GA₃ gibberellic acid, ½ MSMS medium half strength

and 1.19 ± 0.17 cm, respectively; Table 2). Regarding the number of leaves, the largest number of leaves occurred in ½ MS medium + GA₃ (3.76 ± 0.10 leaves), followed by MS medium without growth regulator and MS + 1 mg L⁻¹ GA₃ (2.92 ± 0.12 and 2.25 ± 0.10 leaves, respectively). There was a 100% survival of *G. skinneri* plantlets in the acclimatization phase under greenhouse conditions (Fig. 5b).

Discussion

Micropropagation of orchids has been carried out mainly through conventional culture systems using semisolid medium (Chugh et al. 2009; Bhattacharyya et al. 2017). However, TIS has been implemented as an alternative that involves lower costs, higher multiplication rates, and seeking to achieve a semi-automated micropropagation process (Murthy et al. 2018; Shen et al. 2018; Zhang et al. 2018). *G. skinneri* has been micropropagated in semi-solid culture systems only, with higher production costs associated with the use of the gelling agent (Coello et al. 2010; Coutiño-Cortés et al. 2017). Coello et al. (2010) produced 10.2 shoots per explant using MS medium supplemented with BAP + NAA. However, this methodology was developed in a semi-solid culture system. For their part, Coutiño-Cortés et al. (2017) managed to produce callus only in MS medium supplemented with 3 mg L⁻¹, under the same cultivation system. Particularly, the micropropagation in TIS of *Cattleya* species has been studied for commercial-ornamental purposes, such as the study by Moreira et al. (2013) in *Cattleya walkeriana*, who showed that having a

temporary immersion bioreactor promoted a bigger growth of the aerial part and of the root system, bigger accumulation of dry mass and better control of water loss by the plants. In our study, we implemented a micropropagation protocol for this species in TIS, adjusting almost all of the necessary parameters to guarantee its efficiency. In order to be able to use it for commercial purposes or for the reintroduction and conservation of this species listed as threatened by the Mexican regulations.

There are other factors that should be considered in the optimization of a micropropagation protocol. For example, the composition of the culture, orchids, as epiphytes, require low-nitrate culture media, as is the case of the VW medium (Li et al. 2018). However, in our study, *G. skinneri* showed a better development in MS medium combined with TIS. During micropropagation in TIS, immersion times and frequencies must adapt to the plant species studied, as this parameter largely determines the development and onset of physiological disorders such as hyperhydricity (Georgiev et al. 2014; Mosqueda-Frómata et al. 2017; Murthy et al. 2018). *G. skinneri* showed a higher number of shoots per explant at a lower immersion frequency (2 min each for 4 h). These results are consistent with those reported by Shen and Hsu (2018) for *Phalaenopsis* in TIS, with 10 min/4 h as the best immersion frequency to achieve optimal development. In contrast, Zhang et al. (2018) determined that 3 min/ 6 h was required for the micropropagation of *Bletilla striata* in TIS.

On the other hand, the necessary use of constant subcultures has been reported to increase the multiplication rate of several orchid species, such as *Cymbidium sinense* (Gao et al. 2014) and *Phalaenopsis* sp. (Shen and Hsu 2018). In our study, during the different subcultures *G. skinneri* generated roots without the need to perform a rooting phase. The use of subcultures was also necessary in the micropropagation of *G. skinneri*. However, the generated shoots were small (in terms of length), a problem that must be addressed. This issue has been reported for different orchid species micropropagated in different TISs, such as *Vanilla planifolia* (Ramírez-Mosqueda and Iglesias-Andreu 2016) and *Bletilla striata* (Zhang et al. 2018). The size of explants may be a morphological indicator of development to continue the acclimatization phase and contribute to a higher percentage of survival. In our study, this issue was solved through an elongation phase prior to the acclimatization process. There was a 100% survival of *G. skinneri* plantlets in the acclimatization phase. This is in contrast with studies recommending the use of mycorrhizal fungi to achieve a high percentage of survival of micropropagated shoots of *G. skinneri* (Gutiérrez-Miceli et al. 2008).

In conclusion, this study describes a protocol for the micropropagation of *G. skinneri* under temporary immersion system, particularly in temporary immersion bioreactors (TIB®). This protocol can be used for commercial

purposes. In addition, this can contribute to the conservation and reintroduction of this valuable plant genetic resource.

Author contributions MARM and JJBB conceived and designed research. ORLO and JMG conducted experiments. MARM, RNP, and JJBB analyzed and reviewed the statistical analysis. MARM and JJBB wrote the manuscript. MARM, JJBB, ORLO, RNP, and JMG read and approved the manuscript.

Compliance with ethical standards

Conflict of interest The author(s) declare that they have no conflicting interests.

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