SCIENTIFIC NOTE

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INFLUENCE OF VESSEL TYPE, PHYSICAL STATE OF MEDIUM AND TEMPORARY IMMERSION ON THE MICROPROPAGATION OF THREE *Rhodophiala* SPECIES

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ABSTRACT

Rhodophiala C. Presl (Amaryllidaceae) is a genus of attractive flowering geophytes native to South America. They have ornamental value, but most species are not well- known and have conservation problems. The objective of this study was to optimize a micropropagation process to support the use and preservation of three Chilean native species, *R. montana* (Phil.) Traub, *R. splendens* (Rengifo) Traub., and *R. ananuca* (Phil.) Traub. The research evaluated the feasibility of implementing liquid medium culture and assessed the influence of different tissue culture systems on the shoot production and biomass increment of small bulbs. Three experiments were carried out. The first one determined the influences of flask size and volume of media; the second compared liquid and solid media, and in the third experiment, a temporary immersion system (TIS), and conventional culture in static liquid, shaken liquid and gelled Murashige and Skoog (MS) media were compared. By using larger (350 mL) flasks with higher (50 mL) media volume, 100% more fresh weight of microbulb was obtained that treatment with smaller flasks (45 mL) and media volume (10 mL). In gelled medium, hyperhydricity affected only 5% of explants, while in liquid medium was 16-40%. Survival to acclimatization reached 87-94% for plants from gelled medium; from liquid medium only 38-69%. TIS yielded higher propagation rate (1.9 shoots in 30 d) compared with shaken liquid medium (1.0) ($P < 0.05$) in *R. ananuca* only. Current procedures are appropriate for the support of *ex situ* conservation and germplasm bank establishment.

Key words: TIS, liquid culture, micropropagation, acclimatization, *Rhodophiala*.

INTRODUCTION

The genus *Rhodophiala* C. Presl (Amaryllidaceae), native to South America, comprises 31 bulbous species (Traub, 1956). These wild-growing geophytic plants have high ornamental potential because of their attractive red, pink, yellow or orange flowers. However, most *Rhodophiala* species are vaguely known and grow only in restricted, isolated geographic areas. Furthermore, their survival is endangered because of habitat destruction (Schiappacasse *et al*., 2002).

For both commercial utilization and *ex situ* conservation a fast and efficient vegetative propagation system is needed. However, like other Amaryllidaceae, vegetative multiplication by bulb offsets is very low (Schiappacasse *et al*., 2002; Angulo *et al*., 2003).

When small bulbs of *R. bagnoldii* are cultivated *in vitro* employing solid Murashige and Skoog (1962) (MS) medium, multiple shooting is obtained only when a slight incision in the basal plate is applied to the bulblets (Olate and Bridgen, 2005). It is also feasible to culture *R. montana*, *R. splendens* and *R. ananuca* in gelled MS medium, but the average multiplication rate is quite low (1.5 to 2 to 60 d) (Jara *et al*., 2005). The same authors confirmed that a slight incision in the basal plate of bulblets may induce new shoots, but with considerable variability in number between explants. Treatments with different combinations and concentrations of growth regulators, usually applied in other Amaryllidaceae, have not been successful in improving propagation rates (Jara *et al*., 2005). Therefore, different aspects of the micropropagation process must be adjusted to improve propagation rates, vitroplant quality and *ex vitro* viability.

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In conventional micropropagation processes, vessel type influences the *in vitro* performance of explants by a consequence of differences in microculture humidity and gaseous exchange. In some species, vessel size is a striking factor, greatly affecting microshoot quality and influencing shoot length and shoot number per explant (McClelland and Smith, 1990).

Gelled or liquid medium can drastically change the *in vitro* performance of explants, even when using the same medium formulation, because of differences in microculture humidity and nutrient availability. Even though liquid culture systems can generate some disorders, such as hyperhydricity, they generally encourage faster growth and propagation. Additionally, liquid media are more amenable to automation, as shown by several authors who used the temporary immersion system (TIS). TIS also has additional advantages such as promoting more uniform media and oxygen supply, decreasing apical dominance and stimulating lateral bud growth (Etienne and Berthouly, 2002; Adelberg, 2004; Zhu *et al*., 2005; Ruffoni and Savona, 2005; Ziv, 2005).

This study was focused on evaluating the feasibility of implementing liquid medium culture and on assessing the influence of different tissue culture systems on shoot production, bulb enlargement, hyperhydricity and browning of explants from *R. montana*, *R. splendens* and *R. ananuca*. Different types of flasks, solid or liquid media and TIS were used.

MATERIALS AND METHODS

Origin of plant material: Seeds of *R. montana* (collected from Laguna del Maule, Chile), *R. splendens* (from Vilches Alto, Chile) and *R. ananuca* (from Aguada de Tongoy, Chile) were entered into *in vitro* culture as described by Jara *et al*. (2005), germinated and kept in MS medium with 30 g L^{-1} of sucrose, where microbulbs were formed.

Influence of flask size and medium volume

Microbulbs of *R. montana* and *R. ananuca* were wounded with an incision in the basal plate and cultivated in 45 mL flasks containing 10 mL MS liquid media with 30 g L^{-1} of sucrose without plant growth regulators (45/10, Table 1) and 350 mL flasks containing 50 mL of the same medium (350/50). In both treatments, a hydrophilic cotton disc was used as a support. A single explant was placed in each flask with 25 replicates per treatment. The cultures were kept at 22 ± 2 ºC under 50 μmol m^{-2} s^{-1} of light (fluorescent white light Phillips TLT 40W/54 RS) for 60 d with a 16:8 h photoperiod. The variables evaluated were the number of shoots and the plant and bulb growth indices [(final weight - initial weight)/ initial weight]. Statistical analyses were performed by comparing the means through a Student's t test. A Mann-Whitney test was used for variables having a non-normal distribution. Abnormal plants showing translucent tissues of glassy appearance or brown tissue were classified as hyperhydric and browned individuals, respectively, statistical differences were assessed by Chi Square Test. The significance level for statistical analysis for this and all experiments in this study was $P < 0.05$.

After the experiment was concluded, the remaining nutrient content in the medium was analyzed. N-NO_3 and N-NH_4 were determinate by Kjeldahl method, P by molybdenum blue colorimetric method, K by spectrophotometry of atomic emission and Mg by spectrophotometry of atomic absorption.

Micropropagation in liquid and gelled MS medium

In order to determine the influence of liquid or solid medium on the *in vitro* performance of explants and their

Table 1. Number of shoots, microbulb growth, browning and hyperhydricity *in vitro* of *Rhodophiala* species cultured in two vessel types with different flask size and medium volume.

Treatment	Number of shoots	Plant growth index	Bulb growth index	Bulb weight	Browned explants	Hyperhydric explants
				g	%	%
			R. montana			
45/10	1.5 ± 0.2	5.7 ± 0.7b	1.7 ± 0.3b	0.6 ± 0.1b	64a	7
350/50	2.1 ± 0.4	8.6 ± 1.1a	4.5 ± 0.9a	1.2 ± 0.1a	13b	16
			R. splendens			
45/10	1.3 ± 0.1	7.1 ± 1.5	1.4 ± 0.3b	0.5 ± 0.1b	44	11
350/50	1.6 ± 0.2	8.3 ± 1.5	2.5 ± 0.4a	1.0 ± 0.9a	17	14

Different letters indicate statistical differences (P < 0.05) between treatments within species only. If letters are not displayed, this indicate no significant differences. Values correspond to mean ± standard error.
45/10: 45 mL flasks with 10 mL Murashige and Skoog (MS) liquid media; 350/50: 350 mL flasks with 50 mL MS liquid media. *R. ananuca* was not included in this experiment due to unavailability of plant material.

subsequent acclimatization, two treatments were carried out.

Microbulbs of *R. montana*, *R. splendens* and *R. ananuca* with an incision in the basal plate were cultivated in 350 mL flasks with 50 mL of liquid MS medium supplemented with 30 g L^{-1} of sucrose, without plant growth regulators, with a hydrophilic cotton disc as a support, or in the same type of flask with 50 mL gelled (8 g L^{-1} agar Fluka Chemie AG, Buchs, Switzerland) MS medium plus sucrose. A single explant was placed in each flask, with 20 replicates per treatment. Plants were kept at same conditions indicated earlier. Afterwards, the same variables as in the first experiment were evaluated. Additionally, a subset of five explants from each treatment was used for determining the proportion of dry matter (DM)

For the acclimatization experiment, explants were grown for 30 d inside closed plastic trays in peat/ sand substrate (1:1), fertilized with 50% MS solution. A constant temperature of 22 °C and a 16 h day were maintained in the growth chamber. After that, the boxes were placed for another 30 d in a greenhouse with an average temperature of 21 °C (min. 12 °C; max. 29 °C) with 15 h approximately of day length. Finally, the plants were placed into 1.5 L pots containing a soil/sand substrate (2:1), cultivated for 7 or 8 wk and fertilized every 15 d with a solution of 1 g L^{-1} KNO_3 and 1 g L^{-1} NH_4PO_4.

After each acclimatization step, survival (%), plant weight (g), plant growth index [(final weight of plant after three stages of acclimatization - initial weight *ex vitro*)/ initial weight *ex vitro*], and bulb diameter (mm) were measured. Additionally, correlations between weight reached *in vitro* and *ex vitro* treatments were made.

Statistical analyses were performed by comparing the means through a Student's t test between results from liquid and gelled medium and subsequent acclimatization stages, for each species separately. A Mann-Whitney test was used for variables having a non-normal distribution. Chi square test was used to asses statistical differences on percentage of hyperhydric and browned explant and differences on survival to acclimatization.

Comparing the temporary immersion system and conventional culture in static liquid, shaken liquid and gelled MS media

In this experiment, six treatments were compared. The experimental unit was five micro bulbs each with an incision in the basal plate. In each treatment, three replicates of the experimental unit were used. The temporary immersion system (TIS) was of the "twin-flasks" type (Figure 1A), based on the model described by Etienne and Berthouly (2002). Flasks were 18.2 cm tall and 8.5 cm wide. The influence of media volume on microbulb performance was tested using 20, 40 and 60 mL of medium per explant. The flooding height was 2.2, 3.6 and 5.3 cm, respectively. In all three treatments, one immersion of 3 min every 12 h was programmed. The other three treatments consisted of conventional microbulb culture in static liquid media (1000 mL flask 100 mL^{-1} of medium) with a hydrophilic cotton disc as support, liquid media (same flask type and volume) in orbital shaker (Unimax 2010, Heidolph Instruments, Schwabach, Germany, 100 rpm) and gelled media (same flask type and volume) in 8 g L^{-1} agar. In all cases, the culture medium was MS supplemented with 0.54 μM naftalenacetic acid (NAA), 4.4 μM 6-benzylaminopurine (BAP) and 30 g L^{-1} of sucrose. Experimental design was completely randomized.

After 30 d, the same variables as in experiment one were evaluated using the ANOVA Test. Tukey test was performed for mean comparison. The data were calculated as percentages (hyperhydric and browned explants) and transformed with the arcsine function before carrying out the statistical test. In all cases, Statgraphics Plus 5.0 software was used.

RESULTS AND DISCUSSION

In the first experiment, for both *Rhodophiala* species tested, the utilization of larger flasks with a higher media volume (350/50) did not affect the number of shoots. Independent of treatment, only a few bulbs developed between 2 to 7 new shoots (Figure 1B and 1D). There was no relationship between explant weight and number of shoots ($P = 0.99$; $R^2 = 0.0\%$). Treatment 350/50 significantly increased the bulb growth index (Table 1). The size of flask should have influenced the gaseous components and the humidity of the microenvironment, affecting *in vitro* growth (Kozai *et al*., 1986); however, a previous experiment performed on *Rhodophiala* using 45, 120 and 350 mL flasks with the same volume of medium showed no differences in the growth index (Muñoz, 2006). In the present study, differences in the growth index could be attributed to the availability of nutrients because the concentrations of the nutrients NH_4, P, and K remaining in the medium were higher with the 350/50 treatment than the 45/10 treatment. It is also possible that this effect was due to the dilution of exudates because fewer browned explants were observed with a higher medium volume (Table 1).

When comparing liquid and gelled MS medium, after 60 d of *in vitro* cultivation, similar shoot development was achieved in the three *Rhodophiala* species. On the

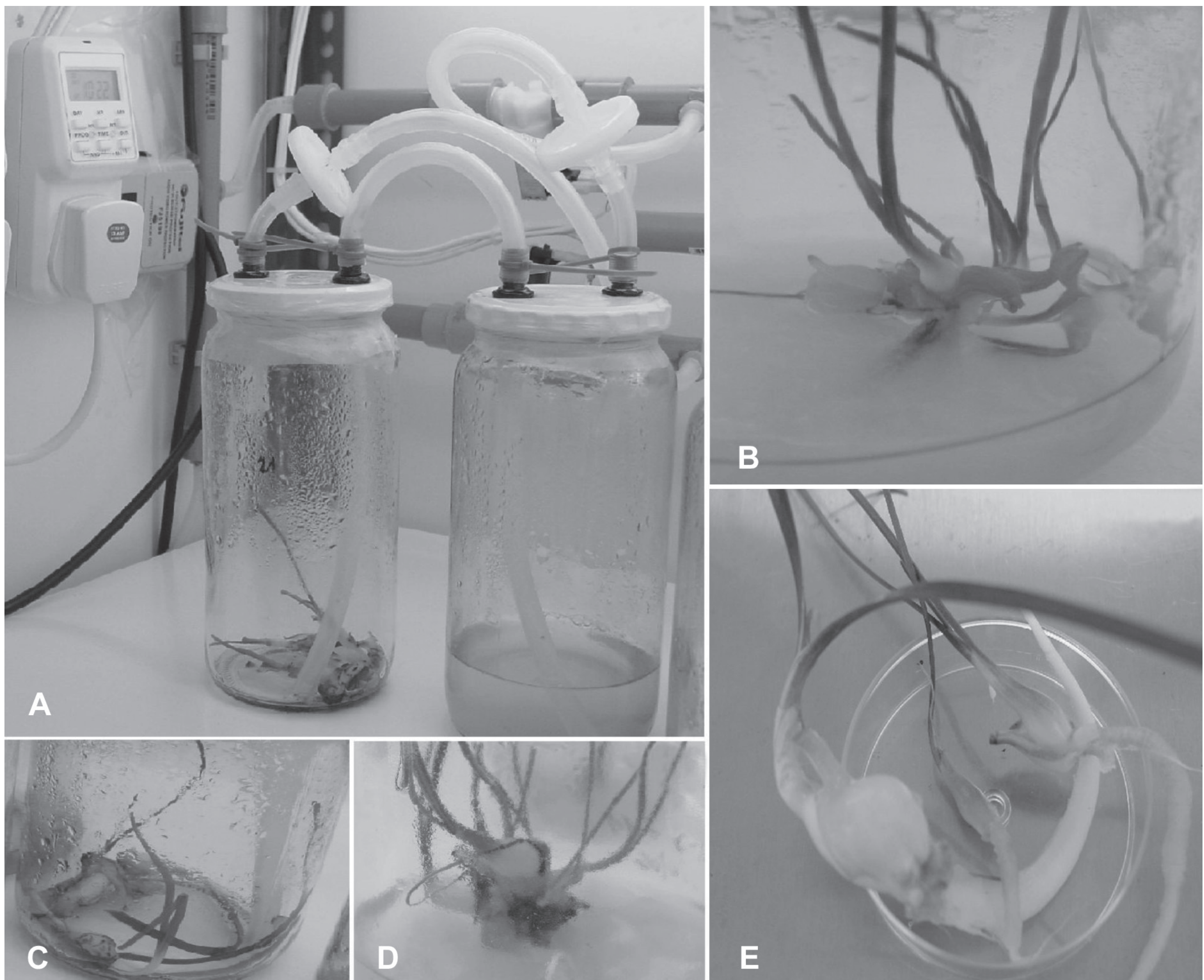

Figure 1. ***In vitro*** **development of** ***Rhodophiala*** **species: A) Temporary immersion system (TIS) type "Twin flask" for** ***Rhodophiala*** **culture. B) Multiple shooting from single explant of** ***R. montana*** **cultured in liquid medium with cotton support. C) Details of explants visualization inside TIS unit. D) Multiple shooting from single explant of** ***R. ananuca*** **cultured in gelled medium. E) Small bulbs harvested from** ***in vitro*** **culture, ready to start acclimatization.**

other hand, the bulb growth index was significantly higher in liquid culture system for *R. montana* and *R. splendens*. In all cases the DM percentage did not differ between treatments, but higher browning in *R. ananuca* and hyperhydricity in *R. splendens* and *R. ananuca* were observed in explants developed in liquid media (Table 2).

After three stages of acclimatization, the survival of *in vitro* grown plants using solid media was high (87-94%), whereas for plants derived from liquid media survival was significantly lower (38% for *R. splendens* and *R. ananuca*). In the Amaryllidaceae, successful acclimatization seems to be related to the weight of bulbs, even for plants coming from liquid medium cultures (Angulo *et al*., 2003). In our work, bulbs for acclimatization weighed between 0.16 and 3.8 g (Figure 1E), but there was no correlation between the *in vitro* bulb weight and the survival of plants coming from liquid media. However, in the three *Rhodophiala* species, all *in vitro* plants classified as hyperhydric died during acclimatization. Mortality could be related to the disruption of hydric regulation and transpiration. Plants from liquid medium cultures, probably well adapted to hydrophytic conditions, were severely affected by *ex vitro* conditions; this effect was especially observed for *R. splendens* and *R. ananuca*.

Plants that survived the acclimatization process

Table 2. Micropropagation, *in vitro* development, and greenhouse acclimatization of three *Rhodophiala* species cultured *in vitro* in solid and liquid media.

Treatment	*In vitro* development								Greenhouse growth		
	Number of shoots	Plant growth index	Bulb growth index	Bulb weight	Browning	Hyperhydric explants	DM	Survival *ex vitro*	Plant weight	Plant growth index	Bulb diameter
				g	%				g		mm
R. montana											
Liquid	1.5 ± 0.3	4.0 ± 0.6a	2.1 ± 1.1a	1.8 ± 0.3a	16	16	14.0 ± 0.7	69	2.0 ± 0.4	1.2 ± 0.4	7.9 ± 0.8
Solid	1.4 ± 0.2	2.9 ± 0.8b	0.8 ± 0.8b	0.8 ± 0.1b	0	5	15.3 ± 1.1	87	1.4 ± 0.3	1.9 ± 0.4	6.4 ± 0.8
R. splendens											
Liquid	1.2 ± 0.1	3.3 ± 0.5	2.1 ± 0.3a	1.4 ± 0.2	35	40a	13.6 ± 0.7	38b	2.4 ± 0.6	1.2 ± 0.4	8.4 ± 0.6b
Solid	1.3 ± 0.2	3.2 ± 0.8	1.1 ± 0.2b	1.1 ± 0.2	11	5b	14.5 ± 2.1	94a	1.5 ± 0.3	2.4 ± 0.5	6.3 ± 0.5a
R. ananuca											
Liquid	1.3 ± 0.2	6.8 ± 0.9	4.3 ± 0.6	0.8 ± 0.1	52a	33a	12.8 ± 1.2	38b	1.7 ± 0.3	0.7 ± 0.2b	7.9 ± 0.3
Solid	2.2 ± 0.6	6.0 ± 0.7	3.1 ± 0.4	0.8 ± 0.1	0b	5b	14.3 ± 0.1	94a	2.0 ± 0.2	2.3 ± 0.3a	8.2 ± 0.3

Different letters indicate statistical differences ($P < 0.05$) between treatments within species only. If letters are not displayed, this indicate no significant differences. Values correspond to mean ± standard error. DM = dry matter.

reached similar final weights (fresh biomass) independent of previous *in vitro* culture conditions (solid or liquid medium). Bulb weight achieved *in vitro* in liquid medium had no relationship ($P = 0.1$; $R^2 = 3.6\%$) with the final plant weight after *ex vitro* culture, suggesting that *in vitro* fresh biomass is not a good predictor of survival and further *ex vitro* growth.

Only one temporary immersion treatment in *R. ananuca* produced more shoots than the liquid shaken medium (Table 3). In the rest of the treatments (TIS, static and gelled media treatments), the response of explants was similar for all species tested (Figure 1C). TIS has resulted in an important improvement in the micropropagation of potatoes (*Solanum tuberosum* L.), banana (*Musa* x *paradisiaca* L.) and yucca as reported by Etienne and Berthouly (2002), but results with bulbous plants have been moderately better or not different when compared to conventional tissue culture systems (e.g., *Charybdis* sp. and *Hippeastrum x chmielii*) (Wawrosch *et al*., 2005; Ruffoni and Savona, 2005; Ilczuk *et al*., 2005).

CONCLUSIONS

When *Rhodophiala* microbulbs were cultivated in gelled media, multiplication rates were low, but *in vitro* plantlets normally grew with a high percentage of survival to acclimatization and adaptation to greenhouse conditions. It is possible to cultivate *Rhodophiala* in conventional liquid media if automation or lower cost process is desired, but additional efforts must be made to control hyperhydricity. Temporal immersion system appears to effectively control hyperhydricity, but it does not have a significant influence on plant propagation rate and *in vitro* bulb growth, compared with gelled medium. These bulbous species show moderate or none response to TIS, probably due to conservative patron of development that favors the growth of mother bulbs instead of daughter bulb initiation, behavior difficult to break, even using different systems that involve differences in microculture humidity, gaseous exchange and availability of medium components.

Current protocols for the micropropagation of *Rhodophiala* are not efficient enough for commercial purposes but could be used for *ex situ* conservation in germplasm banks.

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Table 3. Effect of temporary immersion systems (TIS), static liquid, shaken liquid, and gelled MS media on shoot number and growth of *Rhodophiala* microbulbs.

Treatment	Number of shoots	Plant growth index	Bulb growth index	Browning	Hyperhydricity
				%	%
		R. splendens			
TIS 20	1.3 ± 0.2	0.22 ± 0.05	0.13 ± 0.03	35 ± 24	4 ± 4a
TIS 40	1.1 ± 0.1	0.24 ± 0.02	0.18 ± 0.01	63 ± 9	24 ± 13ab
TIS 60	1.2 ± 0.1	0.22 ± 0.09	0.16 ± 0.07	43 ± 23	38 ± 12ab
Static liquid	1.1 ± 0.1	0.27 ± 0.09	0.17 ± 0.05	31 ± 6	63 ± 9b
Shaken liquid	1.1 ± 0.1	0.33 ± 0.3	0.28 ± 0.30	29 ± 20	62 ± 2b
Gelled	1.0 ± 0.0	0.12 ± 0.01	0.08 ± 0.00	66 ± 18	7 ± 4a
		R. montana			
TIS 20	1.06 ± 0.1	0.06 ± 0.05	0.04 ± 0.03	40 ± 6	18 ± 10a
TIS 40	1.06 ± 0.1	0.16 ± 0.05	0.12 ± 0.03	23 ± 13	17 ± 17a
TIS 60	1.30 ± 0.2	0.14 ± 0.07	0.06 ± 0.03	27 ± 2	0 ± 0a
Static liquid	1.00 ± 0.0	0.08 ± 0.01	0.02 ± 0.01	27 ± 4	13 ± 7a
Gelled	1.20 ± 0.1	0.02 ± 0.03	-0.02 ± 0.02	22 ± 5	18 ± 12a
		R. ananuca			
TIS 20	1.9 ± 0.2a	0.38 ± 0.20	0.22 ± 0.10	6 ± 3	12 ± 12a
TIS 40	1.1 ± 0.1ab	0.20 ± 0.10	0.16 ± 0.09	7 ± 4	25 ± 8a
TIS 60	1.3 ± 0.1ab	0.30 ± 0.06	0.20 ± 0.08	8 ± 4	19 ± 13a
Static liquid	1.6 ± 0.2ab	0.52 ± 0.30	0.18 ± 0.10	8 ± 2	8 ± 4a
Shaken liquid	1.0 ± 0.0b	0.12 ± 0.05	0.10 ± 0.06	0 ± 0	93 ± 7b
Gelled	1.6 ± 0.2ab	0.12 ± 0.03	0.04 ± 0.01	8 ± 2	4 ± 4a

Different letters indicate statistical differences ($P < 0.05$) between treatments within species only. If letters are not displayed, this indicate no significant differences. Values correspond to mean ± standard error.
TIS: temporary immersion system; TIS 20, TIS 40 and TIS 60 mean 20, 40 and 60 mL per explant, respectively.

RESUMEN

Influencia del tipo del recipiente, estado físico del medio e inmersión temporal en la micropropagación de tres especies de *Rhodophiala*. *Rhodophiala* C. Presl (Amaryllidaceae) es un género de geófitas nativas de Sudamérica con un gran potencial ornamental por sus atractivas flores. La mayoría de las especies de este género son poco conocidas y tienen problemas de conservación. El objetivo de este estudio fue optimizar un proceso de micropropagación para sustentar el uso y preservación de tres especies nativas de Chile: *R. montana* (Phil.) Traub, *R. splendens* (Rengifo) Traub y *R. ananuca* (Phil.) Traub. Se evaluó la factibilidad de la implementación del cultivo en medio líquido y se determinó la influencia de diferentes sistemas de cultivo *in vitro* en la formación de brotes e incremento en biomasa de microbulbillos. Se ejecutaron tres experimentos. El primero determinó la influencia del tamaño del frasco y el volumen de medio Murashige y Skoog (MS), el segundo comparó medio MS líquido y sólido, y el tercero comparó un sistema de inmersión temporal (SIT), líquido estático, líquido en agitación y medio MS gelificado. Con frascos de 350 mL con volumen de medio de 50 mL se obtuvo un 100% de mayor peso fresco de bulbo que en frascos de 45 mL con volumen de medio de 10 mL. En medios gelificados, la hiperhidricidad afectó al 5% de los explantes, mientras que en medio líquido fue 16-40%. La supervivencia a la aclimatación alcanzó un 87-94% para plantas provenientes de medio gelificado; para las provenientes de medio líquido llegó a 38-69%. SIT permitió obtener mayor tasa de propagación (1,9 brotes en 30 días), comparado con medio líquido en agitación (1,0 brote) ($P < 0,05$) en *R. ananuca*. Los procedimientos presentados son apropiados para la conservación *ex situ* y el establecimiento de bancos de germoplasma.

Palabras clave: SIT, cultivo en medio líquido, micropropagación, aclimatación, *Rhodophiala*.

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