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RESEARCH PAPER

In vitro bulbing for the propagation of *Traubia modesta* (Amaryllidaceae), a threatened plant endemic to Chile

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Abstract

K. Paredes, C. Delaveau, P. Carrasco, C. Baeza, Freddy Mora, and M.E. Uribe. 2014. *In vitro* bulbing for the propagation of *Traubia modesta* (Amaryllidaceae), a threatened plant endemic to Chile. Cien. Inv. Agr. 41(2): 207-214. Critically endangered, *Traubia modesta* is endemic to Chile and belongs to the family Amaryllidaceae. In this research, a propagation protocol was developed for the *in vitro* cultivation of vegetative organs for this species. The twin scale explants were cultured in a Murashige and Skoog (MS) medium, supplemented with the growth regulators naphthaleneacetic acid (NAA) and 6-benzylaminopurine (BAP) in different concentrations. Plant preservative mixture (PPM) and polyvinyl pyrrolidone (PVP) were used as a biocide and an antioxidant, respectively. The results showed high contamination of the bulbs. There were no significant differences between the treatments with plant growth regulators. A multiplication rate of 1.3 – 2.2 for bulbils was achieved, with an average of 28 bulbils per mother bulb. The natural regeneration rate is approximately 1 - 4 bulbils per mother bulb once a year.

Key words: Bulbing, critically endangered species, *In vitro* propagation, *Traubia modesta*, twin scales.

Introduction

The genus *Traubia* is endemic to Chile and belongs to the Amaryllidaceae family. The genus contains a single species, *T. modesta* (Phil.) Ravenna (Ravenna, 2003). This endangered plant from central Chile is locally known as "añañuca modesta" and "añañuca blanca". This bulbous plant is classified as endangered (Ravenna *et al*., 1998). It inhabits the coastal zones and hills from the south Coquimbo Region (29° 54′ S, 71° 15′ W) to the Libertador Bernardo O'Higgins Region (34° 10′ S, 70° 43′ W) (Baeza *et al.,* 2009). Five populations are known to exist between Los Molles in the north and Quilpué in the south (Los Molles-Pichidangui, Altos de Petorca y Alicahue, Bosque de Zapallar, Laguna Verde and Cordillera el Melón). These locations are badly damaged by urban development (Novoa, 2007).

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T. modesta is a plant characterized by its small size. It measures 15-20 cm in height, with linear or slightly swollen leaves, which are dry or non present at the time of blooming. Flowers measure 15-20 mm in length and are pure white or white with purple veins near towards to the base, with a purple line along the back of the tepals. The fruits are capsules that contain black-shiny seeds that are flattened, rounded and with membranous edges. In its natural habitat in Chile, *T. modesta* blooms from January to May (Baeza *et al.,* 2009).

Studies related to vegetative propagation of *T. modesta* are unknown; however, it is known that geophytes from Amaryllidaceae have a low natural propagation rate that ranges from approximately zero to eight bulbs per mother bulb (Santos *et al.,* 1998; Schiappacasse *et al*., 2002; Angulo *et al*., 2003). Conventional propagation techniques for ornamental purposes have been used for the recovery of other species of endangered bulbous plants. For example, *Herbertia lahue* (Mol.) Goldbl. is a species from which Morales *et al.* (2007) obtained 1.13 bulbils per bulb, which is within the expected range found for other Chilean geophyte species such as *Calydorea xiphioides* Poepp. and *Leucocoryne coquimbensis* F. Phil (Kim *et al.,* 1998), which produced 1.5 and 1.1 to 1.6 bulbils per mother bulb, respectively (Morales *et al.,* 2007*).*

In studies performed keep carried out by Schiappacasse *et al.* (2002), 18 species of Chilean geophytes with ornamental potential were propagated. In these studies, a maximum of 8.6 bulbils per initial bulb were obtained by using bulb division as the method for vegetative propagation. When performing the twin-scaling separation technique with *Rhodophiala bagnoldii* (Herb.) Traub, the number increased to 30 bulbils per mother bulb, demonstrating the potential of these techniques.

To compare the karyotype of *T. modesta* with a species of the same family, Baeza *et al.* (2009) conducted a cytogenetic study. This study defined the chromosome morphology of *T. modesta*, which is very different from the other species in the family.

In vitro tissue culture is a very useful biotechnological technique for the production and recovery of species that are threatened or endangered. Several studies have been conducted on the propagation of bulbous species. For example, Ozel *et al.* (2008), for example, developed an efficient *in vitro* multiplication method for *Ornithogalum ulophyllum* Hand.-Mazz. using the twin-scaling technique. For *Cyrtanthus clavatus* (L'Hér.) R.A. Dyer and *Cyrtanthus spiralis* Burch. ex Ker Gawl., the twin scales were cultured in a liquid medium to increase the bulb production rate under aseptic conditions (Morán *et al.,* 2003). However, Angulo *et al.* (2003) followed the protocols for the micropropagation of *Cyrtanthus loddigesianus* (Herb.) R.A. Dyer and *Cyrtanthus speciosus* (L.f.) Traub., and they demonstrated a high efficiency using *in vitro* culture protocols.

In Chile, *in vitro* production of bulbils from different species of the genus *Rhodophiala* of the Amaryllidaceae family has occurred, with bulb production ranging from 8 to 57 bulbils per mother bulb, which is higher than the natural regeneration rate (Ferrando, 2002).

The keep aim of this research was to establish a protocol for the *in vitro* culture of *T. modesta* bulbils from the twin scales. The effects of growth regulators on the multiplication of bulbils of *T. modesta* was determined to help with the proper propagation of the species. The populations of *T. modesta* are highly reduced and very vulnerable (Baeza *et al*., 2009), and the results of this study will contribute to the conservation of the species.

Materials and methods

Plant material and culture conditions

The bulbs of *T. modesta* used in this trial were collected in La Ligua, a region of Valparaíso, Chile (32° 26' S, 71° 13' W), in sites with increasing urbanization. Surface sterilization of the collected bulbs was performed before beginning *in vitro* cultures. Roots, the upper-third of the stem, and

dry outer scales were removed. The samples were washed with antibacterial soap and a brush under tap water for 10 min. Subsequently, the samples were placed in a solution with 1.5 g L^{-1} of Polyben 50 WP (Anasac™) and 200 mg L^{-1} of streptomycin sulfate (Calbiochem™) for 24 h. After this period, under a laminar-flow chamber with constant agitation, the bulbs were rinsed in sterile distilled water for 3 min to begin the process of surface asepsis. The bulbs were immersed in a 70% ethanol solution for 2 min, followed by 3 rinses with sterile distilled water of 3, 4 and 5 min. They were further disinfected in a 40% (v/v) commercial bleach solution with 2.5% active bleach with two drops of common dish soap (Quix®) for 15 min, which was followed with a triple rinse with sterile distilled water. Bulbs were kept in sterile distilled water during the introduction of the scales to the growth medium to prevent the desiccation of the material.

The growth medium for this experiment was the MS medium (Murashige and Skoog, 1962), supplemented with 30 g L^{-1} of sucrose. To prevent tissue oxidation, 0.5 g L^{-1} of polyvinyl pyrrolidone (PVP, Calbiochem™) was used, and as a biocide, 1 mL of plant preservative mixture (PPM™) was added. Once the growth solution was prepared and before sterilization, the pH was adjusted to 5.8. The medium was gelated with 8 g L^{-1} of plant agar (Duchefa). Flasks or test tubes with the growth medium were autoclaved for 20 min at 1 atm of pressure at 121 °C.

Explants were placed in either test tubes with 10 mL or in glass flasks with 30 mL of growth medium. Explants were incubated in a growth chamber at 25±1 ºC and 55% relative humidity with a 16 h photoperiod with the cold light at an intensity of 40 µmol m^{-2} sec^{-1}.

Establishment and induction stage

Once the bulbs were aseptic, they were sectioned into eight longitudinal slices that were separated in the twin scale explants, with a portion of the basal disk. These scales were vertically inserted into the tubes with the MS growth medium. To identify the optimal concentration, different concentrations of different growth regulators were added to the medium. These treatments were as follows: (A) without growth regulators, (B) supplemented with 1.0 mg L^{-1} of 6-benzylaminopurine (BAP) and 0.5 mg L^{-1} of naphthaleneacetic acid (NAA), (C) 0.5 mg L^{-1} of BAP and 1.0 mg L^{-1} of NAA, and (D) 1.0 mg L^{-1} of BAP and 1.0 mg L^{-1} of NAA.

The experimental unit was a test tube with one explant; treatment A had 56 explants, and treatments B, C and D each had 64 explants. The experiment was a completely randomized design. In the first three subcultures of this phase, the number of contaminated, necrotic and viable explants was counted. In the final count, those explants that were not contaminated were selected for the multiplication phase.

In the statistical analyses, a Generalized Linear Model (deviation analysis) was used, with a binomial distribution of the response variables (viable, contaminated and necrotic explants) and a logarithmic link function. Analyses were performed with the SAS® GENMOD software procedure (SAS Institute, Cary, North Carolina, USA).

Multiplication phase

Uncontaminated viable explants obtained from the establishment and induction phase were cultured in fresh media in 5.5 cm diameter by 6.5 cm high glass flasks with 30 mL MS growth medium for the following treatments: (A) without growth regulators, (B) supplemented with 3.0 mg L^{-1} of BAP and 0.5 mg L^{-1} of NAA, (C) 2.0 mg L^{-1} of BAP and 1.0 mg L^{-1} of NAA, and (D) 2.0 mg L^{-1} of BAP and 2.0 mg L^{-1} of NAA.

The number of new bulbils per treatment was assessed. The data were subjected to statistical analyses, accounting for a Poisson distribution for the number of new bulbils variable. The General-

ized Linear Model under the GENMOD procedure (SAS™) was used with a logarithmic link function (Myers *et al*., 2002; Mora *et al*., 2010).

Results and discussion

Establishment and induction stage

The twin scales of *T. modesta* were highly contaminated because they came directly from their natural habitat, with contamination reaching 50% to 80% of the total number of explants per treatment. This contamination is associated with the activity of fungi and bacteria in the internal tissues of the plant material (Alvarado, 1998). According to Pierik (1990), although the plant material is surface-sterilized before culturing, complete sterilization is not possible. The analyses of deviations in the subcultures showed significant differences among the treatments for percentages of viability, contamination and necrosis of the explants (Table 1).

Table 1. Deviation analyses (Chi-square test) of viable, contaminated and necrotic explants in the *in vitro* bulbing experiment with *Traubia modesta*.

		Chi-square		
Source	DF	Viable	Contaminated	Necrotic
Intercept				
Treatment	3	25.45*	21.34*	5.91 ns
Time	3	101.96*	45.63*	101.64*

*P≤0.05, ns=not significant, and DF=degrees of freedom.

The high percentage of contamination observed during the establishment phase in this study has been previously recognized, and such contamination represents the main challenge for micropropagation (Smith *et al.,* 1999). For example, the bulbs of *Narcissus* sp. (Amaryllidaceae) contain a mucilage in which pathogenic organisms live that are difficult to eliminate with surface sterilization (Sochacki and Orlikowska, 2005). Chang *et al.* (2003) found that the marshy habitat that *Zantedeschia* sp. (Araceae) inhabited was favorable for the development of bacteria and decomposer fungi in the tubers, which caused great losses for the commercial propagation of this species. Additionally, they maintained that the endogenous contamination was the greatest problem with the propagation of the tubers. Thermotherapy and fungicides in combination with immersions in NaOCl solutions can lower survival rates and eliminate contamination (Bruyn *et al*., 1992).

According to Slabbert *et al.* (1993), to avoid the contamination problem with *Crinum macowanii* Baker, a selection of bulbs was performed. The bulbs were collected and placed in the greenhouse. An induction phase was started, and the material that presented some degree of contamination could be discarded (20% to 40% of the scales from *C. macowanii*). Moreover, Ault (1995) found 90% of the contaminated plant material in the establishment phase for *Eucomis autumnalis*. For *Sprekelia formosissima*, Cázarez *et al. (*2010) found better results following previous trials with disinfection methodologies, with 89.1% of the explants axenic.

The results from the present study agree with those obtained by Ferrando (2002) who found that the contamination percentages were so high that it was concluded that any asepsis method used for the three species of *Rhodophiala* would be favorable for the multiplication of the species. In the current study, the contamination was only by fungi because the bacterial contamination could be efficiently controlled with streptomycin and PPM as biocides. The decision to include an antibiotic, such as streptomycin, was made following Ferrando (2002), who recommended using these substances because of the endogenous bulb contamination. Streptomycin sulfate is widely used as antibiotic for *in vitro* cultures. However, PPM has proved to be efficient in controlling exogenous fungal and bacterial contamination in *in vitro* cultures (Digonzelli *et al.,* 2005). With *Amaryllis* species, low contamination rates of the explants were achieved with different concentrations of PPM (Smith *et al.,* 1999*)*. With *R. bifida,* Rodrigo *et al.* (2006) found that the seeds treated with PPM were aseptic and had low levels of contamination, with no effect of PPM on later development or morphology.

The percentage of viable explants varied among the different hormone treatments and with the time grown in these media. For viability, a maximum of 42.2% of the explants were viable in treatment D (Table 2).

Table 2. Analyses of the significant differences among treatments for viable and contaminated explants of *Traubia modesta*.

	Viable		Contaminated	
Contrast	Chi^2	$P > Chi^2$	Chi^2	$P > Chi^2$
A versus B	11.23	0.0008*	10.91	0.0010*
A versus C	0.79	0.3743	0.26	0.6081
A versus D	21.35	<.0001*	13.98	0.0002*
B versus C	6.54	0.0105*	8.38	0.0038*
B versus D	1.77	0.1836	0.21	0.6490
C versus D	15.05	0.0001*	11.20	0.0008*

*$P \leq 0.05$.

Another disadvantage of working with bulbous species is the oxidation and the necrosis of the tissues that occurs. Oxidation and darkening of the *in vitro* cultured explants is produced by the reaction of free radicals, as well as the oxidation of phenolic compounds (Ferrando, 2002; Azofeifa, 2009). In this study, a rapid oxidation of the tissues in the zone where the sections were performed was observed. The maximum percentage of material lost by this oxidation reached 20.3%. In the case of *Rhodophiala,* Ferrando (2002) used antioxidant baths of citric and ascorbic acids, as well as drops of sodium diethylthiocarbamate, but the results were not as expected and almost all the material was lost. With *Hippeastrum* spp., necrosis and browning was observed in the third and fourth subcultures (Seabrock and Cumming, 1977).

In summary, with the contamination and subsequent oxidation leading to necrosis, the maximum survival of the treated explants varied between 5 and 15% in the later phases of the culture.

Multiplication phase

The scales used during the establishment and induction process had low rates of development of new shoots, so those with the most regeneration were selected for the multiplication phase. These were transferred to growth media with different concentrations of growth regulators; greater than those used in the establishment and induction stage. These scales produced new bulbs (Table 3) with new shoot formations present in all treatments (Figure 1).

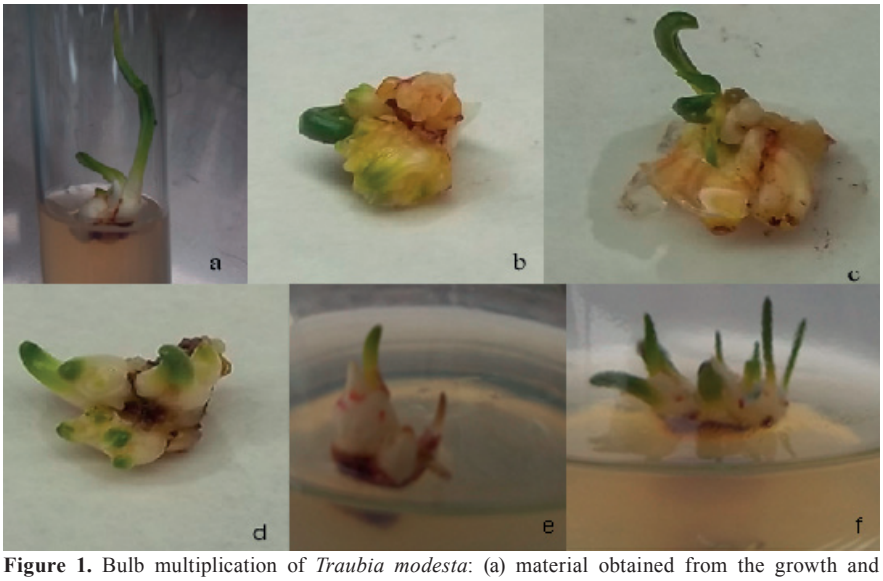

Figure 1. Bulb multiplication of *Traubia modesta*: (a) material obtained from the growth and induction phases, (b, c and d) new formations in the multiplication phase, and (e and f) new bulbils of *Traubia modesta* produced *in vitro* with growth regulator in treatment D (2.0 mg L^{-1} of BAP and 2.0 mg L^{-1} of NAA).

Table 3. Number of new bulbs of *Traubia modesta* per treatment during three subcultures.

SCV (d)	TREAT	N	NNB	IC
30	A	14	1.29	±0.53
	B	16	1.31	±0.54
	C	21	1.29	±0.39
	D	15	1.73	±0.44
80	A	14	1.21	±0.41
	B	16	1.94	±1.18
	C	21	1.52	±0.55
	D	15	2.47	±0.98
120	A	14	1.64	±1.58
	B	16	2.50	±2.13
	C	21	1.33	±0.44
	D	15	2.20	±0.92

N=Number of individuals, NNB=Number of new bulbs, TREAT=treatment, SCV=subculture, d=days, and IC=confidence interval of 95%. A. without growth regulators, B. 3.0 mg L^{-1} of BAP and 0.5 mg L^{-1} of NAA, C. 2.0 mg L^{-1} of BAP and 1.0 mg L^{-1} of NAA, and D. 2.0 mg L^{-1} of BAP and 2.0 mg L^{-1} of NAA.

According to the deviation analyses, there were no significant differences among the treatments (Chi-square = 4.01 and the associated probability of 0.26; Poisson distribution in a Generalized Linear Model). Therefore, the treatments can be used interchangeably. However, the treatment without growth regulators would be ideal because of the decreased cultivation costs and the minimal treatment of the vegetal material (Uribe *et al.,* 2011).

The results from the current study agree with those obtained for other species of the Amaryllidaceae family. For example, in the case of *R. montana*, Jara *et al*. (2007) found a shoot regeneration percentage of cultured twin scales of less than 20%. The average number of bulbs obtained in all treatments was 28 per mother bulb, which is more than seven-fold the natural regeneration rate. For *Hippeastrum* spp. hybrids (Seabrook and Cumming, 1977), there was greater production of shoots in a growth medium without growth regulators. The same result was found for *Crinum macowanii* (Slabbert *et al.,* 1993) and for the beginning cultivation phase of *Eucomis comosa* and *Eucomis zambesiaca* (Ault, 1995).

Regarding the micropropagation of Amaryllidaceae, several studies emphasized the necessity of determining the optimal concentration of growth regulators required by each species in each phase of the *in vitro* culture. There are species that need higher concentrations of cytokinins (Cázarez *et al.,* 2010; Uranbey*,* 2010), whereas others require auxins (Piña-Escutia *et al.,* 2010) in equivalent concentrations (Azadi and Khosh-Khui, 2007). Furthermore, there are species that require no addition of growth regulators. In the present study, however, the low availability of plant material did not allow for studies on the interactions between hormones. However, it is recommended that such studies are conducted with *T. modesta* and that these studies should include other types of growth regulators at varied concentrations with different types of growth media, among other factors.

In summary, for *T. modesta*, it is possible to obtain large-scale production of bulbs. A bulbil multiplication rate of 1.3 to 2.2 and an average of 28 bulbs were obtained per mother bulb in all treatments, which is more than seven-fold the natural regeneration rate. According to the results, it is recommended that treatments prior to *in vitro* cultures be developed to obtain axenic cultures more successfully, in addition to counting on a larger number of bulbs

Resumen

K. Paredes, C. Delaveau, P. Carrasco, C. Baeza, Freddy Mora y M.E. Uribe. 2014. Formación de bulbos *in vitro* de *Traubia modesta* (Amaryllidaceae), una planta amenazada endémica de Chile. Cien. Inv. Agr. 41(2):207-214. *Traubia modesta* pertenece a la familia Amaryllidaceae y es endémica de Chile, que se encuentra en la categoría de "en peligro" de extinción. En esta investigación se desarrolló un protocolo de propagación de la especie a través del cultivo *in vitro* de órganos vegetativos. Se utilizó la técnica de escamas gemelas cultivadas en medio MS suplementado con ANA y BAP en distintas concentraciones, se adicionó PPM como biocida y PVP como compuesto antioxidante. Los resultados obtenidos muestran una alta contaminación en los bulbos. No hubo diferencias significativas entre los tratamientos con reguladores del crecimiento vegetal. Se logró una tasa de multiplicación de microbulbillos de 1,3 a 2,2, donde el promedio de bulbos obtenidos en todos los tratamientos por bulbo madre fue de 28. La regeneración natural es alrededor de 1 a 4 unidades por bulbo madre una vez al año.

Palabras clave: Bulbificación, escamas gemelas, especie en peligro de extinción, propagación *in vitro*, *Traubia modesta.*

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