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AN EFFECTIVE *IN VITRO* SLOW GROWTH PROTOCOL FOR CONSERVATION OF THE ORCHID *Epidendrum chlorocorymbos* SCHLTR.

[UN PROTOLOCO EFECTIVO DE CRECIMIENTO IN VITRO PARA LA CONSERVACIÓN DE LA ORQUÍDEA *Epidendrum chlorocorymbos* SCHLTR.]

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SUMMARY

Efficient slow growth protocol for the orchid *E. chlorocorymbos* Schltr. was developed using *in vitro* conservation studies. Seedlings were placed in MS culture medium and a 2x3x3 factorial design applied to evaluate the effects of supplementing the medium with mannitol (0, 1, 2 and 3%), sucrose (0, 1, 2 and 3%) or sorbitol (0, 1, 2 and 3%). Experimental conditions were a 16:8 h photoperiod, 23 ± 2 °C temperature and 50-80% relative humidity. At 6 months, the best treatment was MS medium at half ionic strength with 1% sorbitol. The culture was also free of contamination. This resulted in slow growth and normal morphology during maintenance and successful growth afterwards. Shoots were subsequently recovered, multiplied and rooted on MS medium with sucrose 3% without addition of growth regulators.

Key words: *in vitro* storage; mannitol; sorbitol; *Epidendrum chlorocorymbos* Schltr.

RESUMEN

Se desarrolló un protocolo de lento crecimiento para la conservación *in vitro* de la orquidea E. *chlorocorymbos* Schltr. Las plántulas se colocaron en un medio de cultivo con MS y bajo un diseño factorial 2x3x3 para evaluar los efectos de la suplementar el medio con manitol (0,1,2 y 3%), sacarosa (0,1,2 y 3%) o sorbitol (0,1,2 y 3%). Para las condiciones experimentales se utilizó un fotoperiodo de 16:8 h, una temperatura de 23± 2 °C y se utilizó una humedad relativa entre 50-80%. A los 6 meses, el mejor tratamiento fue el medio MS a la mitad de su fuerza iónica con sorbitol al 1%. El cultivo estuvo libre de contaminación. Esto resulto en un crecimiento lento y morfología normal durante el mantenimiento y posteriormente un crecimiento exitoso. Los brotes recuperados subsecuentemente, se multiplicaron y se enraizaron sobre el medio MS con 3% de sacarosa sin la adición de reguladores de crecimiento.

Palabras clave: Almacen *In vitro;* Manitol,Sorbitol; *Epidendrum chlorocorymbos* Schltr.

INTRODUCTION

The family Orchidaceae is one of the groups most diverse of plants, consisting of ~35,000 species under 800 genera (Singh *et al*., 2007). Orchids are distributed on all continents (except Antarctica), but its greater diversity is concentrated in tropical regions. Mexico has about 1260 species and 170 genera (Hágsater *et al*., 2005). Among the genera of orchids, Epidendrum as the largest genus of orchids with over 1000 species, many of which occur in great abundance in Central America (Chen *et al*., 2002). *Epidendrum chlorocorymbos* Schltr. is an orchid found throughout the Yucatan Peninsula (Carnevali *et al*., 2001), has been reported as a medicinal plant use for cholesterol, sore ears (Burgos-Hernández, 2009). As occurs with orchids in many other locations in Mexico, this species experience heavy exploitation, leading to possible decreases in their native populations. In situ conservation of orchid species is very difficult because of the relatively slow growth of orchids and low germination rates which requires symbiotic relationships with mycorrhizal fungi in natural habitats (Godo *et al*., 2010). One alternative for preventing irreparable loss of biodiversity is use of in vitro conservation of plant germplasm through using slow growth procedures or cryopreservation. Slow *in vitro* growth is usually achieved by using substances such as mannitol and sorbitol to reduce medium osmotic potential (Grout, 1991), a low

concentration of nutrients (Engelmann, 1991). Slow growth storage via *in vitro* cultures has been reported in many species such as *Elettaria cardamomum* (Tyagi *et al*., 2009), *Plumbago indica* Lim (Chaeoensub and Phansiri, 2004), garlic cultivars (Hassan *et al.*, 2007), *Cynara scolymus* (Bekhett and Usama, 2007), *Veronica multifida* spp capsellicarpa (Holobiuc *et al*., 2008), *Drosophyllum lusitanicum* (Gonçalves and Romano, 2007); strawberry (Hassan and Bekheet, 2008), *Deutzia scabra* Thunb (Gabr and Sayed, 2010). Although none has yet been reported for *in vitro* conservation of *Epidendrum chlorocorymbos* Schltr. The present study objective was to evaluate in vitro technique for conservation this orchid specie for induce in slow-growth in an effort to develop genetic diversity conservation protocol.

MATERIAL AND METHODS

Vegetal material and disinfection.

Green capsules with seeds were collected from *Epidendrum chlorocorymbos* Schltr in the region of Escarcega, Campeche state, Mexico.

Capsule treatment and sterilization.

Capsules were placed in a drier at 23 ± 2 ºC for 15 days to allow seeds to reach maturity. Mature capsules were washed with a detergent solution and rinsed with sterile distilled water. The capsules were then placed in a solution (1% commercial sodium hypochlorite and 1 drop of Tween 20) for 10 min, under constant agitation. The washed capsules were dipped in pure alcohol, quickly passed through a flame, the alcohol allowed to completely burn off and the capsules and tweezers left to cool. Once cooled, the sterile capsule was cut in half longitudinally with a sterile scalpel. The seeds extracted from the capsules were distributed over the media culture were added drops of water to separate and distribute in each container (Magenta boxes).

Culture medium and germination conditions.

Epidendrum chlorocorymbos Schltr seeds were germinated in semi-solid medium composed of MS medium (Murashige and Skoog, 1962), basal salt and vitamins supplemented with 30 g l^{-1} sucrose, 2.2 g l^{-1} gelrite without growth regulators. The seeds were placed in and incubated for 12 weeks. See culture conditions below.

Slow growth treatments.

At three months of age, *in vitro* germinated seedlings with two leaves, 1-2 roots and heights of (ca. 5 mm) *Epidendrum chlorocorymbos* Schltr were placed in the different slow growth treatments. A 2x3x3 factorial design was applied to evaluate the effect of each culture medium's ionic strength, carbon source (osmotic agent) and carbon source concentration on number shoots and number leaves formed after six months in vitro conservation. Two treatments were used as control (MS full ionic strength and half ionic strength without carbon source). All treatments are described in Table 1. For all treatments seedlings were placed in test tubes (20 X 150 mm) containing 20 ml medium. Each culture tube received one explant enclosed with polypropylene caps and covered with cling film.

Table 1.Treatments evaluated for slow growth in the orchid *Epidendrum chlorocorymbos* Schltr.

Treatment code	MS. Ionic Strength	Carbon Source		
		Sucrose (%)	Mannitol (%)	Sorbitol (%)
TFSA1	100%	1	0	0
TFSA2	100%	2	0	0
TFSA3	100%	3	0	0
THSA1	50%	1	0	0
THSA2	50%	2	0	0
THSA3	50%	3	0	0
TFM1	100%	0	1	0
TFM2	100%	0	2	0
TFM3	100%	0	3	0
THM1	50%	0	1	0
THM2	50%	0	2	0
THM3	50%	0	3	0
TFS1	100%	0	0	1
TFS2	100%	0	0	2
TFS3	100%	0	0	3
THS1	50%	0	0	1
THS2	50%	0	0	2
THS3	50%	0	0	3
TCON1	100%	0	0	0
TCON2	50%	0	0	0

Data recording and statistical analyses.

Each treatment had four replicates. Data were recorded after six months of conservation in slow growth treatments. Data were subjected to analyses of variance (ANOVA) and means were compared by Tukey test using Stat graphics plus version 5.1 program.

Table 2. Effect of treatments of slow growth on shoot and leaves formed in plantlets of *Epidendrum chlorocorymbos* Schltr.

Treatment code	Response variables Assessment at six months of culture	
	Number Shoots	Number Leaves
TFSA1	13.25± 1.50^{ab}	48.00± 4.24^{b}
TFSA2	4.50±1.29^{cd}	13.50±3.87^{e}
TFSA3	12.00±1.63^{b}	36.00±4.89^{c}
THSA1	13.25±1.50^{ab}	39.70±4.50^{c}
THSA2	15.00±2.58^{ab}	37.75±14.56^{c}
THSA3	21.00±3.46^{a}	53.00±10.39^{a}
TFM1	0.00±0.00^{e}	0.00±0.00^{h}
TFM2	0.00±0.00^{e}	0.00±0.00^{h}
TFM3	0.00±0.00^{e}	0.25±0.50^{h}
THM1	0.00±0.00^{e}	0.75±0.50^{gh}
THM2	0.00±0.00^{e}	0.05±0.57^{gh}
THM3	0.00±0.00^{e}	0.05±0.57^{gh}
TFS1	2.50±0.50^{d}	9.00±1.63^{f}
TFS2	3.50±0.57^{d}	8.00±3.36^{fg}
TFS3	6.50±1.00^{c}	18.25±0.50^{de}
THS1	2.50±1.00^{d}	8.50±3.00^{fg}
THS2	7.25±1.50^{bc}	21.75±4.50^{d}
THS3	12.50±3.00^{b}	32.25±12.09^{c}
TCON1	0.00±0.00^{e}	0.00±0.00^{h}
TCON2	0.00±0.00^{e}	0.00±0.00^{h}

Multiplication after slow growth.

To test the viability at the end of the six-month experimental period in slow growth conditions, survival and growth were evaluated. shoots from selected treatment were removed from culture tube and washed under running tap water to remove the adhering gelling agent. Shoots were transferred to fresh MS medium with macro and micro elements and vitamins, containing 3% sucrose and solidified with 0.2% Gel-Rite.

Culture conditions of germination, slow growth and multiplication. All the chemicals used for preparing the media were of analytical grade (Sigma). In all cases, medium pH was adjusted to 5.7 with KOH 0.1 N and/or HCl 0.1 N before sterilizing in an autoclave at 121°C and 1.06 kg cm−2 pressure for 20 min. All experiments were incubated a 23 ± 2 ºC, under a 16:8 (light:dark) photoperiod using Philips cool white fluorescent tubes of 1500 lux.

RESULTS AND DISCUSSION

Effect of treatments of slow growth on shoot and leaves formed in plantlets of *Epidendrum chlorocorymbos* Schltr was evaluated at six months.

Values are mean ± SE of 4 cultures for each treatment. Values superscripted with the same letter in each column are not significantly different on the basis of ANOVA Stat graphics plus.

Effect carbon source in slow growth

The beneficial effect of mannitol for in vitro conservation has been previously reported in *Discorea alata* (1.5%) Borges *et al*., 2004; *Plumbago indica* (2%) (Chaeoensub and Phansiri, 2004); *Drosophyllum lusitanicum* (2%) Gonçalves and Romano, 2007; *Veronica multifida* ssp (3 and 6%) Holobiuc *et al*., 2008. In this it work observed that mannitol treatments produced *Epidendrum chlorocorymbos* seedlings death, this explain since it has been reported the toxic effects of mannitol in

vitro conservation, Lata *et al*., 2010 reported that 2% manitol was not adequate for in vitro conservation of *Podophyllum peltatum*, Loureiro da Silva *et al*., 2011 observed that 1-3 % mannitol negatively affect causing low rate of recovered shoots after storage of *Piper hispidernervum* and *Piper aduncum* shoots. However, the lethal concentration seems to be species dependent (Loureiro da Silva *et al*., 2011), and storage time can influence, as reported Bekhett and Usama, 2007, in *Cynara scolymus* that the presence of mannitol in culture medium had a retardant effect on the growth and development of all cultures and data of reveal that high percentage of survival and healthy shoot bud cultures were recorded after the first three months of storage on medium containing 4% mannitol, but survival and healthy shoot cultures were sharply decreased after six months of storage.

The treatment with both sucrose and sorbitol resulted in the mass multiplication of shoots in treatments. Due to the formation of multiple shoots from the initial seedling it was not possible to measure the plant length variation and therefore this parameter was not considered. When analyzing the variables of shoots and leaves formed significant difference was found between the carbon sources, p-value are in Table 3 and 4 respectively. In Fig.1a was observed that sorbitol produced less shoots than sucrose, at 1 and 2% (Fig.1b). Similar results was observed on number leaves (Fig. 2). These results agree with those found for diminished growth reported in the presence of sorbitol. Although the protocol established for *E. chlorocorymbos* is less expensive since only use 1% while in *C. scolymus* was necessary at 4% (Bekhett and Usama, 2007), sorbitol 0.2 M has also been found to be suitable as an osmotic agent for the conservation of strawberry germplasm (Hasan and Bekheet, 2008), and 0.16 M sorbitol is reported to preserve African violet sprouts (Moges *et al*., 2003). In *E. chlorocorymbos* was observed that lack of a carbon source produced abnormal morphology and plantlets did not develop in these treatments.

Effect ionic strength in slow growth

Lowering the content in mineral elements of culture medium resulted in terms of number of shoots and leaves no significant difference between the ionic strength of MS medium (Table 3 and 4 respectively), however, in the graphics of interaction is observed that sorbitol gives less number of shoots and leaves with 50% MS (Fig. 3). This agrees with the results reported in slow growth conservation of cardamom (Tyagi *et al*., 2009), in vitro conservation coconut embryos (Sukendah and Cedo, 2005).

Table 3. Analysis of Variance for shoots - Sums square of Type III

Source	Sum of square	DF	Mean square	Ratio F	p-value
Main effects					
A:MS:Ionic strength	11.6806	1	11.6806	3.28	0.0755
B:Carbon source	2410.36	2	1205.18	338.00	0.0000
C: Concentration	93.5278	2	46.7639	13.12	0.0000
Interactions					
AB	341.861	2	170.931	47.94	0.0000
AC	28.5278	2	14.2639	4.00	0.0236
BC	107.889	4	26.9722	7.56	0.0001
Waste	206.806	58	3.56561		
Total (Corrected)	990.625	71			

F ratios are based on the residual mean square error

Table 4. Analysis of Variance for leaves - Sums square of Type III

Source	Sum of square	DF	Mean square	Ratio F	p-value
Main effects					
A:MS:Ionic strength	26.8889	1	26.8889	0.62	0.4340
B:Carbon source	21627.1	2	10813.6	249.59	0.0000
C: Concentration	1166.03	2	583.014	13.46	0.0000
Interactions					
AB	1955.44	2	977.722	22.575	0.0000
AC	397.028	2	198.514	4.58	0.0142
BC	1451.72	4	362.931	8.38	0.0000
Waste	2512.89	58	43.3257		
Total (Corrected)	29137.1	71			

F ratios are based on the residual mean square error

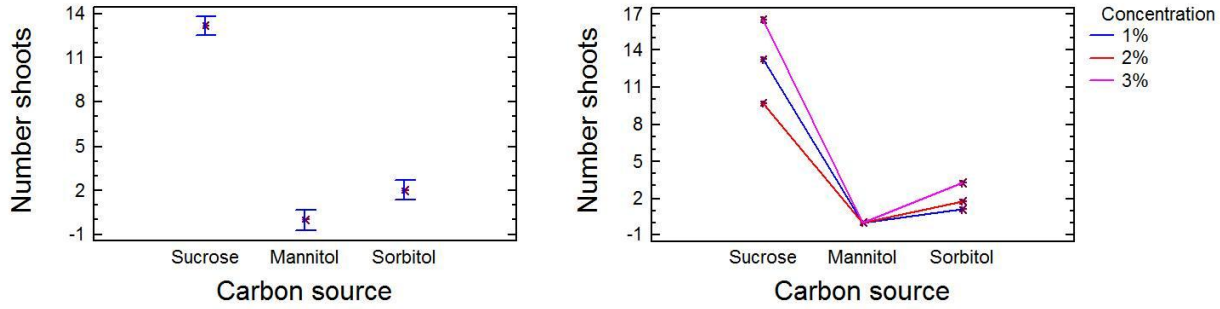

Figure 1.Graphics of number of shoots by *E. chlorocorymbos.* (a) Interval means and percentages Tukey of effect carbon source; (b) Interaction between factors: carbon source and concentration

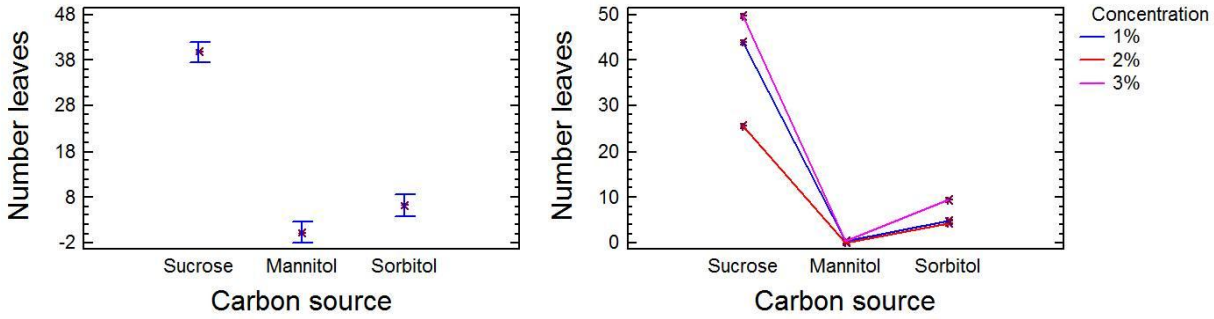

Figure 2.Graphics of number of leaves by *E. chlorocorymbos.* (a) Interval means and percentages Tukey of effect carbon source; (b) Interaction between factors: carbon source and concentration.

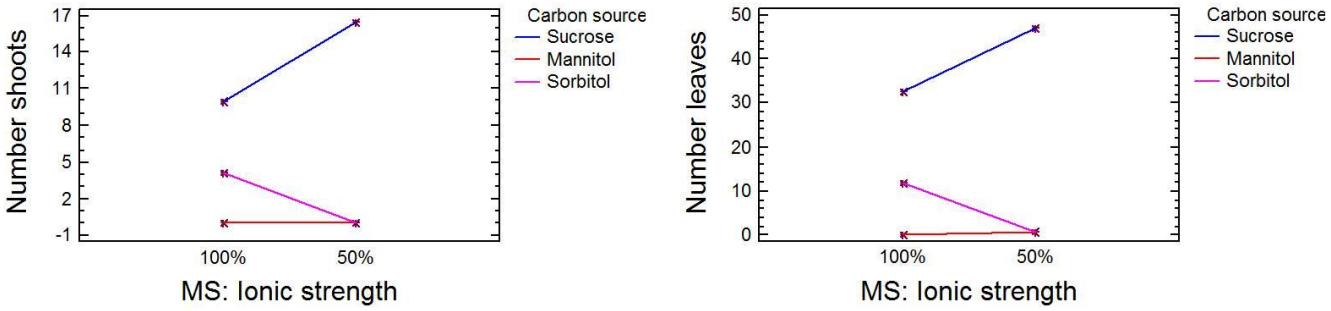

Figure 3.Graphics of Interaction between factors: Ionic strength and carbon source. Number of shoots. (b) Number leaves

Slow growth protocol for *E. chlorocorymbos*

A protocol *in vitro* slow growth for conservation of *E. chlorocorymbos* was established by use of osmotic regulators such as sorbitol, this regulator added to the culture medium reduce hydric potential, consequently restricting water availability to the plantlets and lowering mineral uptake (Shibli *et al.*, 2006). In contrast to most germplasm conservation protocols, low temperatures were unnecessary in the present study; cooling raises conservation costs also the conservation mediated by osmotics may be more economical than cryopreservation Tehrim and Sajid, 2011. At six months, it was clear that the treatments with the lowest shoots and leaves development were also those with the lowest plantlet development. These results of this study indicate that the optimum treatment is 50% of ionic strength MS supplemented with 1% sorbitol, as this gives the lowest amount of growth (Fig 4a) whilst the maximum growth occurred in MS with 3% sucrose (4b).

Multiplication after slow growth conservation

Survival after the conservation period was 100% in shoots of *E. chlorocorymbos* in the treatment THS1. Growth and rooting occurred in both cases and no abnormalities were observed (Figure 5).

CONCLUSIONS

Although research on the development of conservation techniques has been done for numerous plant species, this is a first report for in vitro conservation for *Epidendrum chlorocorymbos* Schltr germplasm. Growth reduction was attained by modifying culture medium composition through addition of the osmotic regulator sorbitol. Optimal conditions for slow growth were 1% sorbitol and 50% of ionic strength MS. Establishing the proper conditions for slow growth in orchids will aid in preserving their germplasm for the purpose of later reintroduction and sustainable use.

Figure 4. Shoots of *Epidendrum chlorocorymbos* Schltr after six month of slow growth conditions. (a) In treatment THS1 (MS 50%, sorbitol 1%). (b) In the treatment TFSA3 (MS 100% 3% sucrose).

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Figure 5. Seddlings regenerated of *Epidendrum chlorocorymbos* Schltr after six months of conservation in slow growth conditions in treatment THS1

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