

Sexual micropropagation of the critically endangered Christmas orchid *Masdevallia towarensis*, Aragua, Venezuela

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SUMMARY

The critically endangered Christmas orchid *Masdevallia towarensis* is endemic to cloud forest within the state of Aragua (Colonia Tovar) northern Venezuela. Its propagation has been little studied, particularly regarding *in vitro* cultivation techniques. In an attempt to asymbiotically and sexually micropropagate this autochthonous species, seeds were used from indehiscent capsules obtained from nurseries in the vicinity of areas where this orchid naturally occurs. The effect of Murashige and Skoog nutrition medium, and different concentrations of thidiazuron, benzyladenine (BA) and naphthaleneacetic acid (NAA) on seed germination were evaluated. *In vitro* asymbiotic germination was characterized by initial swelling of seeds, increase of embryo size and formation of protocorm-like bodies (PLBs), similar to those naturally generated during symbiotic germination. After three months, the PLBs started to sprout leaves and roots to form seedlings. In all treatments, the formation of PLBs was observed, although in higher number when using the combination BA (2.0 mg/l) + NAA (1.5 mg/l). A protocol was obtained that will enable mass propagation by means of asymbiotic germination of seeds *in vitro*.

BACKGROUND

Christmas orchid *Masdevallia towarensis* (rchb. F) Luer is an endemic species of northern Venezuela. It inhabits a restricted area of the Pico Codazzi National Monument, particularly in Colonia Tovar (state of Aragua), in cloud forests between 1,700 and 2,300 m altitude (Llamozas *et al.* 2003). It is mostly rupicolous (i.e. growing on rocks) but also grows less commonly as an epiphyte. It is frequently and traditionally sold in the local market of Colonia Tovar during the Christmas period as it blooms in November through January. It has been exported by commercial nurseries due to the ornamental value of its white coloured flowers. It is listed as 'Critical Endangered (CR)' due to its restricted distribution, deforestation of its cloud forest habitat, the reduced population size and ongoing exploitation as an ornamental plant (Llamozas *et al.* 2003).

There is very little information regarding propagation of this orchid, a greater knowledge of which could greatly contribute to its conservation, whether by reintroduction into its' natural habitat or mass commercial production to reduce collection pressure on extant wild populations. *In vitro* cultivation techniques may constitute a powerful tool because of the potential mass and fast propagation of the species. It may allow the *in vitro* and asymbiotic germination of sexual seeds; when sexual seeds are used the genetic variability is maintained, they require very little space for handling of seedlings, they serve as support for genetic improvement and they allow the *in vitro* conservation of interesting genotypes (Arditti & Ernst 1993, Rao 1997, Teixeira da Silva 2003).

This investigation was conducted during 2008-2009 and focused on the development of an efficient protocol for asymbiotic *in vitro* sexual propagation to allow mass propagation of *M. towarensis* and longer-term reintroduction into

suitable protected natural habitats while maintaining genetic variability.

ACTION

Seed preparation: After flowering, seeds from indehiscent capsules (fruits) were obtained from nurseries from the area where they naturally grow. Initially, the indehiscent capsules were classified in accordance to their size and weight, so as to indicate which capsules contained the seeds that would best germinate *in vitro*, a key aspect which has been already reported (Damon *et al.* 2004). Capsules were only superficially disinfected, since, as they are indehiscent, their seeds were considered unlikely to be contaminated. Disinfection was made by washing the material with soapy running water. Later, and under a laminar flow chamber, capsules were submerged in 70% v/v ethanol for 3 min, and then in commercial bleach at 20% v/v (sodium hypochlorite at 5.25% as active ingredient) for 5 min. Finally, they were rinsed four times with sterile distilled water.

***In vitro* trials:** Seeds aseptically extracted from the capsules were planted in the various media. Capsules were cut at one end and the seeds uniformly distributed in Petri dishes containing Murashige and Skoog (1962) nutrition medium, at half concentration, supplemented with vitamins, citric acid, sucrose, and Phytigel® (as the gelling agent) and pH adjusted to 5.4 ± 0.5 (Hurtado & Merino 1987, Serna 1999). Also, different concentrations of the following growth regulators were evaluated: Thidiazuron (TDZ),

benzyladenine (BA) and Naphthaleneacetic acid (NAA).

The four growth mediums tested were: 1) basic nutrition medium (without growth regulators); 2) basic medium with 1 mg/l of TDZ; 3) basic medium with 0.05 of BA, and 4) basic medium with a combination of 2 mg/l of BA + 1.5 mg/l of NAA. There were five replicates of each treatment (growth medium).

The Petri dishes were then placed in a climate room maintained at $21 (\pm 2)^\circ\text{C}$, darkness during the first two weeks, and a light intensity of $32.5 \text{ umol.m}^{-2} \text{ s}^{-1}$ for 12 hours a day; thereafter the experiment ran for 4.5 months where seedlings were obtained.

CONSEQUENCES

The investigation generated a simple procedure for the disinfection of indehiscent capsules that resulted in successful germination of seeds under *in vitro* conditions. A minimum size ($\geq 2 \text{ cm}$) and weight ($\geq 750 \text{ mg}$) were determined for the capsules to be used.

In all treatments, the formation of PLBs was observed. The largest number of protocorm-like structures and, finally, seedlings, occurred when the BA (2 mg/l) and NAA (1.5 mg/l) combination was added to the basic medium, obtaining an efficiency of 50 seedlings produced per fruit (capsule) over a period of 4.5 to 5 months. The effects of growth regulators on production of PLBs and seedling formation are summarised in Table 1.

Table 1. Effects of growth regulators on production of protocorm-like bodies (PLBs) and seedling formation in *Masdevallia towarensis* under *in vitro* conditions.

Treatments	Mean \pm SE	
	PLBs	Seedlings/jar
Basic nutrition medium	6.17 ± 1.47	4.69 ± 1.03
1 mg/l ⁻¹ TDZ	2.50 ± 1.04	0.00
0.05 mg/l ⁻¹ BA	2.10 ± 0.75	1.99 ± 0.90
2.0+1.5 mg/l ⁻¹ BA+ANA	11.00 ± 1.96	8.50 ± 1.36

Asymbiotic *in vitro* germination was characterized by initial swelling of seeds, increase of size of the embryo and formation of protocorm-like bodies (PLBs), similar to those naturally generated during symbiotic germination. Later, the formation of foliar primordia and roots and the development of seedlings (Fig. 1) were initiated.

Conclusions: Obtaining a protocol, as described, for the generation of *M. towarensis* seedlings will enable the mass production of plants for conservation purposes (including potential reintroductions into their natural habitat where populations have been depleted by collection), as well as maintaining genetic variability. Importantly, they will become commercially available (legal sale), thus hopefully alleviating the ongoing exploitation of natural populations.

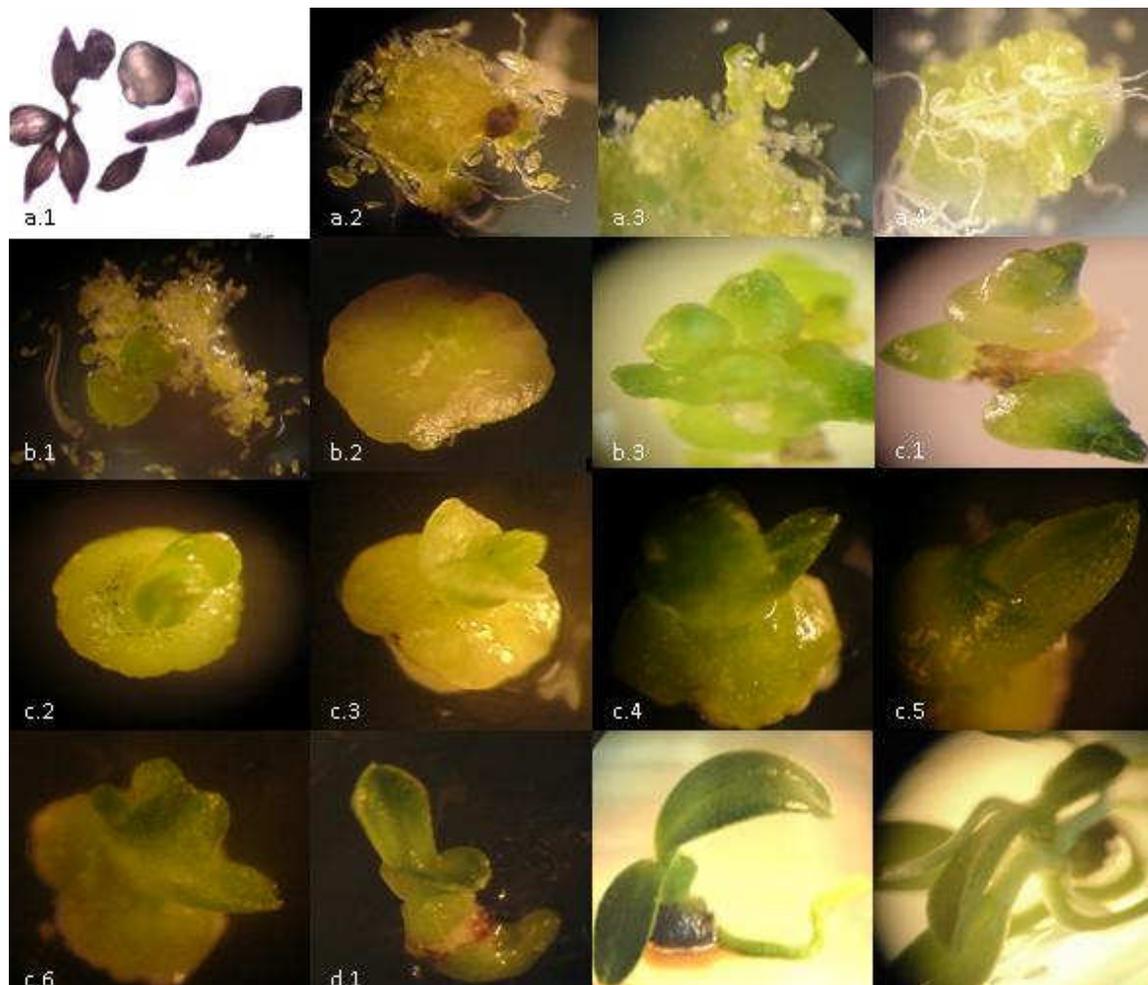


Figure 1. Phenological stages in sexual asymbiotic micropropagation of *Masdevallia towarensis* under *in vitro* conditions. **a** (1-4): germination of seed; **b** (1-3): formation of protocorm or PLBs; **c** (1-6): leaf differentiation; **d** (1-3): protocorm and rhizoids disappearance and the formation of the real roots.

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