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Asymbiotic germination of South African *Holothrix* (Orchidaceae): a successful breeding experiment to prepare repatriation

Maksim I. Antipin, Elena A. Labunskaya & Vladimir V. Choob

Summary: Three *Holothrix* species were germinated in vitro on ½ MS and Malmgren's media and underwent a two-stage growing. Protocorms, primary shoots and tubers of root origin were obtained. The most successful morphogenesis occurred on Malmgren's medium. Plantlets grown on ½ MS medium predominantly demonstrated a development of primary shoots, whereas plantlets transferred from ½ MS to Malmgren's medium developed prominent tubers.

Keywords: Holothrix, in vitro culture, nature conservation, repatriation

The genus *Holothrix* Rich. ex Lindl. (Orchidaceae) was first described in 1835 by John Lindley, who based his description on an earlier work of Louis Richard published in 1818. According to modern estimates, the genus *Holothrix* includes up to 50–60 species distributed in Africa, Arabia and Socotra Island (*H. socotrana*). South Africa holds about one third of the total number of species within the genus (Pridgeon 2001).

Plants of the genus *Holothrix* are either terrestrial or lithophytic orchids with small, ovoid, subterranean tubers of root origin, developing one or two oval or orbicular, often hairy, basal leaves spread flat on the ground. In some species (*H. thodei* Rolfe) the leaves wither away just before anthesis. The flowering season for the majority of species in *Holothrix* is spring and summer. Scapes are erect, unbranched, with or without sheathing leaves. The inflorescence is racemose (simple indeterminate spike). Flowers are small, sessile or shortly stalked, petals entire or fimbriate, the labellum is often lobed, with a spur. Considering the flower structure in *Holothrix*, Vogel (1954) proposed that its most probable pollinators could be hawk moths. Despite of this assumption, there are no reliable data on the pollination of *Holothrix* in the wild up to date (Van Der Cingel 2001). All the species of *Holothrix* demonstrate a pronounced dormancy during the dry season, when the aerial parts of the plant desiccate and die off.

The genus *Holothrix* is usually referred to as belonging to subfamily Orchidoideae, tribe Orchideae. Molecular data of late 1990s have though revealed some ambiguities in the phylogenetic relationships of *Holothrix*. The ITS-based phylogenetic tree (Internal transcribed spacer in rDNA), obtained by Douzery et al. (1999), placed *Holothrix* in subtribe Habenariinae, supporting the earlier viewpoint of Dressler (1993). In spite of this, Bateman et al. (2003) argued against the previous hypothesis, bringing forward the opinion that *Holothrix* should even be excluded from tribe Orchideae. Nevertheless, using the combined markers of nuclear ITS, mitochondrial *cox1* and plastid intron *rp116*, it was shown that genus *Holothrix* may be included into the tribe Orchideae, but could not be affiliated neither to Orchidinae nor to Habenariinae (Inda et al. 2010, 2012). The divergence time of *Holothrix* clade (with *Satyrium, Stenoglottis* and former Diseae included) and the clade of all the other Orchideae, estimated according to

the molecular clock model, was probably Oligocene, shortly after the common ancestor of both clades had appeared (Inda et al. 2012).

Many species of *Holothrix* are endemics with quite small distribution ranges, inhabiting the so called 'hot spots' of biodiversity (Myers et al. 2000). The ever-increasing anthropogenic pressure on these areas may lead to total extinction of certain species. *Ex situ* cultivation, including cultivation in botanical gardens using modern laboratory techniques, provides one of the best chances to conserve and consequently reintroduce endangered species. Still until now, attempts at asymbiotic propagation *in vitro* were performed only for a critically small percentage of African terrestrial orchid species (La Croix & La Croix 1997).

Until very recently, *Holothrix* species were hardly ever presented in living collections of botanical gardens. Very few attempts to cultivate these terrestrial orchids are documented. Having mostly minute, inconspicuous flowers, plants of the genus drew little attention of orchid lovers. Another obstacle in cultivation is the specific ecology of *Holothrix*, which is very different from widely grown tropical epiphytic orchids. Yet, both biology and appearance of *Holothrix* give it a second chance of *ex situ* cultivation by another group of plant enthusiasts, commonly referred to as 'bulb growers'. Amateur collecting of geophytes with ornamental leaves of bizarre shape and surface is now increasing its popularity. Adapted to arid climate, *Holothrix* species with their flat, hairy, fleshy leaves are a classic example of geophyllic plants *sensu* Eller & Grobbelaar (1982). Thus, *Holothrix* species are undeservingly neglected plants with a certain future commercial potential. Developing germination, propagation and cultivation protocols for this plant group may be useful for biodiversity conservation programs and commercial plant growing.

Materials and methods

Seed source and sterilization protocol

Three species of *Holothrix* were planted: *H. burchellii* Rchb. f., *H. secunda* Rchb. f., *H. scopularia* Rchb. f. Seeds were obtained from commercial suppliers (Silverhill Seeds and Lifestyle Seeds, South Africa).

Seeds were surface sterilized in 1.5 ml microtubes (Eppendorf) with 10% commercial bleach solution containing at least 0.5% of sodium hypochlorite. To increase seed wettability, 0.1% (v/v) surfactant (Triton X-100) was also added to the sterilization solution. Seeds were stirred in the sterilizing solution for 5 minutes, then the solution was removed and seeds were rinsed three times with sterile water. To avoid seed loss when removing the liquid, seeds were precipitated at low speed (1000 rpm) for 1 minute by Minispin Eppendorf centrifuge. Rinsed seeds were then injected onto agar media with a small amount of sterile water.

Culture media and culture conditions

Two media, ½ MS medium (Murashige & Scoog 1962) and Malmgren modified terrestrial orchid medium (Malmgren 1996), were used for seed germination. Authors started the experiment with a medium composed of ½ MS salts supplemented with 0.05% of activated charcoal, sugar (10 g/l), glycine (2 mg/l), *myo*-inositol (100 mg/l), nicotinic acid (0.5 mg/l) and pyridoxine chloride (0.5 mg/l). The medium was buffered with 500 mg/l MES (free acid) and solidified with 0.8% agar (further – ½ MS medium). The presence of activated charcoal allows to decrease growth inhibition caused by the leaching oxidized phenolic compounds (Van Waes

Table 1. Malmgren's medium (modified by authors).

Compound	C, mg/l
Calcium phosphate tribasic ($Ca_3(PO_4)_2$)	70
Magnesium sulphate ($MgSO_4 * 7\ H_2O$)	70
Potassium phosphate monobasic (KH_2PO_4)	75
Sucrose	10000
Agar	8000
Activated charcoal	500
Raw turnip (swede)	10 pieces 1 cm^3 each
Raw potato	10 pieces 1 cm^3 each
Aminoplasmal™ E*	5 ml

*Used as the sterile commercial stock manufactured by B. Braun Melsungen.

1987; Waterman & Mole 1994; Teixeria et al. 1994; Miyoshi & Mii 1995). Later, authors tried agar-solidified Malmgren's medium, reportedly producing good results with terrestrial orchids (Malmgren 1996), modifying the protocol slightly (Table 1).

Both media were dispensed to sterile plastic Petri dishes (diameter 40 mm). A dual-phase protocol was used to promote seed germination on Malmgren's medium. It consisted of a solid Malmgren's medium and a layer of the liquid medium with the same content (without agar supplement). This protocol was similar to Thompson's protocol used for *Disa* propagation (Thompson et al. 2003, 2006). Petri dishes were wrapped with plastic film to prevent evaporation and additionally with aluminium foil to create continuous darkness. Germinating seeds were incubated at conditions of a subtropical greenhouse (20–25°C at daytime and 12–16°C at night).

Results and discussion

Effect of growing on ½ MS medium

The seeds of *H. burchellii* and *H. secunda* started to germinate and to form distinct protocorms about 1 month after sowing. From 60 to 90 days the primary shoots appeared (Fig. 1), and then the seedlings were transferred onto tubes with fresh ½ MS medium and placed at diffuse sunlight.

Two months later, the growth rate of protocorms and seedlings considerably slowed down. Protocorms and basal parts of seedlings turned brown. Authors attributed the protocorm browning to possible accumulation of phenolic compounds in the medium (Miyoshi & Mii 1995). Moreover, the plants grown on ½ MS did not emerge any roots neither from shoots nor from protocorms.

Some of the plantlets and protocorms were then transferred onto Malmgren's medium which was worked out specially for terrestrial orchids (Malmgren 1996). The rest of the plants was transferred onto fresh ½ MS medium and cultivated at diffuse artificial light. Five months later, the plants grown on ½ MS medium had green shoots with well-developed leaves, but no detectable adventitious roots were formed.

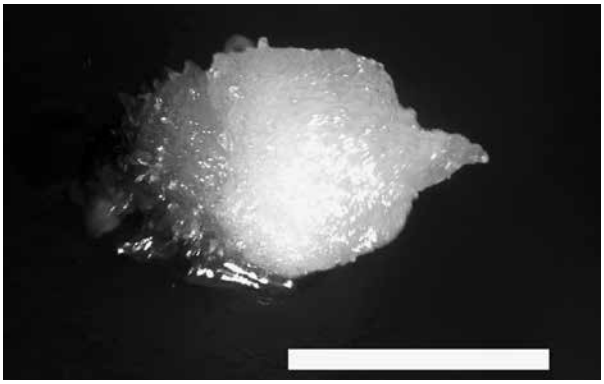

Figure 1. Protocorm of *Holothrix secunda* 2 months after germination on ½ MS medium. Scale bar = 1 mm.

Two-stage growing on ½ MS and Malmgren's media

Authors hypothesize that ½ MS medium is too rich in nitrates and ammonium. These inorganic salts may be an inappropriate source of nitrogen for *Holothrix* seedlings. Thus, Malmgren's medium would be preferential because of organic source of nitrogen in this medium (it includes some amino acids instead of the inorganic nitrogen (Malmgren 1996)). Additionally, Malmgren's medium was supplemented by activated charcoal that has high absorption capacity and is often used for removing of inhibitory substances in culture media (Thomas 2008) and for decreasing of phenolic oxidation (Teixeria et al. 1994).

The rootless green plants and protocorms were thus transferred from ½ MS onto Malmgren's medium, which according to the authors' hypothesis would benefit further rhizogenesis. Within the first month after transplanting, the primary green shoots demonstrated an increased growth and the formation of adventitious roots. Three months later, plants developed tubers of considerable size (Fig. 2). The epidermis of some tubers had differentiated multiple trichoblasts, indicating the root nature of these organs (see Whigham et al. 2008).

Tubers of terrestrial orchids serve as dormant survival structures over summer-drought periods (Dixon 1991). Plants usually develop tubers before the end of wet season (period of their

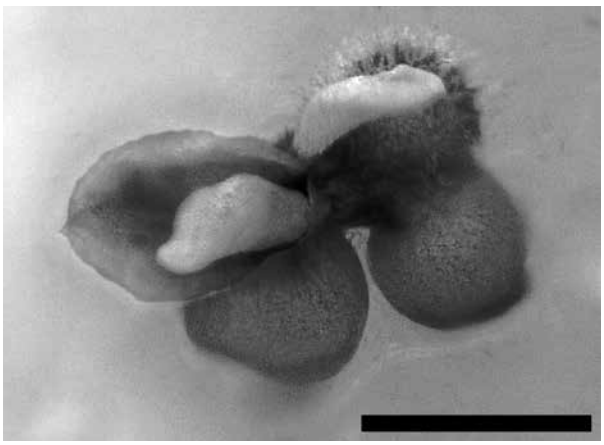

Figure 2. Primary shoot and tubers of *Holothrix secunda* after 4 months of two-stage growing on ½ MS and Malmgren's medium. One of the tubers has multiple trichomes differentiated. Scale bar = 5 mm.

growth), as it was shown for the Australian terrestrial orchid, *Pterostylis sanguinea* (Debeljak et al. 2002). Tuberization may depend on different factors, such as jasmonic acid (Debeljak et al. 2002), sugars (Mohamed-Yasseen et al. 1994) or photoperiod (Martínez-García et al. 2002). However, it is probable that certain orchids do not respond to some of the factors mentioned above: Debeljak et al. (2002) report that high concentration of sucrose had no effect on tuberization in *Pterostylis sanguinea*.

The sucrose content in ½ MS and Malmgren's media was approximately the same (10 g/l). Presumably, the presence of sucrose in Malmgren's medium could not be the key factor of transition to tuberization. In comparison with ½ MS, Malmgren's medium uses natural components (potato, turnip, pineapple) with unidentifiable contents, disputably responsible for better growth and viability of protocorms, juvenile and mature plants (Malmgren 1996). It is possible that some hormones, other growth regulators or secondary metabolites from natural components may stimulate the formation of tubers in orchids. Another possible reason is the difference in nitrogen availability of these two media that was discussed above.

If transferred at the stage of globular protocorm, plantlets slightly increased in size during the first month, but then they stopped their growth, probably entering physiological dormancy. There are several evidences of protocorm dormancy known for certain species of orchids (see Batygina et al. 2003). Some authors describe these dormant structures formed from the globular protocorms as pretuberoids (Szendrák 1997).

Effect of growing on Malmgren's medium

Seeds of *Holothrix scopularia* were incubated on Malmgren's medium using dual-phase protocol (see Materials and Methods). Germination started approximately 40 days after sowing and primary shoots began to develop about 6 weeks after sowing. At this stage, plants were transferred into 100 ml flasks with the solid Malmgren's medium. 6–7 weeks after transplanting, plantlets started forming microtubers (Fig. 3).

Conclusion

Three *Holothrix* species were germinated *in vitro*. They formed a primary shoot, some of them generated tubers. The best results were obtained in single stage Malmgren's medium. Modified ½ MS medium yielded less success: the surviving plants did not develop any adventitious root. Apparently, auxin supplement could help to solve this problem. Adding of auxins and cytokinins is a usual treatment, when seedlings of orchids are planted on MS (Basker & Narmatha Bai 2010; Abraham et al. 2012; Mohanty et al. 2012). Another future challenge is to find the conditions that trigger the growth of dormant protocorms / pretuberoids. Probably, a certain hormone combinaton or variation in medium's sugar content could help to solve this problem. As all *Holothrix* species undergo a dormant period during a dry season, moisture availability may appear to be an essential signal for breaking the dormancy. Consequently, rising the water potential of medium or appliance of the dual-phase method (Thompson et al. 2003, 2006) could change their state.

Better results achieved on Malmgren's medium could possibly be aligned with adding of natural products with unidentifiable content (turnip in Malmgren's protocol), which agrees with other publications, where authors used media with addition of coconut water (Puchooa 2004; Baque et al. 2011) or banana pulp (Aktar et al. 2008).

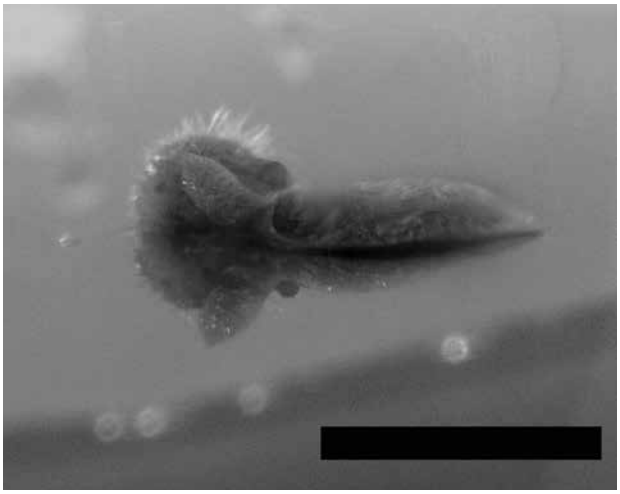

Figure 3. Plantlet of *Holothrix scopularia* grown on Malmgren's medium 18 weeks after sowing according to dual-phase protocol. Primary shoot and developing microtuber with root trichomes. Scale bar = 5 mm.

Tuber formation started 4 months after sowing, which is considerably earlier than in many other terrestrial orchids of Mediterranean climates (Kitsaki et al. 2004; Debeljak et al. 2002). Further investigations are needed to prove that this could be an adaptation to a very short humid season within the arid habitats of the genus.

The results obtained by authors can help to approach the challenge of growing *in vitro* of other species of *Holothrix*, focusing on endemic, critically endangered, endangered and rare species.

References

Abraham S., Augustine J. & Thomas D. (2012): Asymbiotic seed germination and in vitro conservation of *Coelogyne nervosa* A.Rich., an endemic orchid to Western Ghats. – Physiol. Molec. Biol. **18**(3): 245–251.

Aktar S., Nasiruddin K.M. & Hossain K. (2008): Effects of different media and organic additives interaction on *in vitro* regeneration of *Dendrobium* orchid. – J. Agric. Rural Dev. **6**(1–2): 69–74.

Baque M.A., Shin Y.-K., Elshmari T., Lee E.-J.& Paek K.-Y. (2011): Effect of light quality, sucrose and coconut water concentration on the micropropagation of *Calanthe* hybrids ('Bukduseong' × 'Hyesung' and 'Chunkwang' × 'Hyesung'). – Austral. J. Crop Sci. **5**(10): 1247–1254.

Basker S. & Narmatha Bai V. (2010): *In vitro* propagation of an epiphytic and rare orchid *Eria bambusifolia* Lindl. – Res. Biotechnol. **1**(1): 15–20.

Bateman R.M., Hollingsworth P.M., Preston J., Luo Y.-B., Pridgeon A.M. & Chase M.W. (2003): Molecular phylogenetics and evolution of Orchidinae and selected Habenariinae (Orchidaceae). – Bot. J. Linn. Soc. **142**(1): 1–40.

Batygina T.B., Bragina E.A.& Vasilyeva V.E. (2003): The reproductive system and germination in orchids. – Acta Biol. Cracov., Ser. Bot. **45**(2): 21–34.

Debeljak N., Regvar M., Dixon K.W. & Sivasithamparam K. (2002): Induction of tuberisation *in vitro* with jasmonic acid and sucrose in an Australian terrestrial orchid, *Pterostylis sanguinea.* – Pl. Growth Regulat. **36**(3): 253–260.

Dixon K. (1991): Seeder/clonal concepts in Western Australian orchids. – In: Wells T.C.E. & Willems J.H. [eds]: Population ecology of terrestrial Orchids: 111–123. – The Hague, Netherlands: SPB Academic Publishing.

Douzery E. J. P, Pridgeon A. M., Kores P., Linder H. P., Kurzweil H. & Chase M. W. (1999): Molecular phylogenetics of Diseae (Orchidaceae): a contribution from nuclear ribosomal ITS sequences. – Amer. J. Bot. **86**(6): 887–899.

Dressler R. L. (1993). Phylogeny and classification of the orchid family. – Portland, Oregon: Timber Press.

Eller B. M. & Grobbelaar N. (1982): Geophylly: consequences for *Ledebouria ovatifolia* in its natural habitat. – J. Exp. Bot. **33**(2): 366–375.

Inda L. A., Pimentel M. & Chase M. W. (2010): Contribution of mitochondrial cox1 intron sequences to the phylogenetics of tribe Orchideae (Orchidaceae): do the distribution and sequence of this intron tell us something about its evolution? – Taxon **59**(4): 1053–1064.

Inda L. A., Pimentel M. & Chase M. W. (2012): Phylogenetics of tribe Orchideae (Orchidaceae: Orchidoideae) based on combined DNA matrices: inferences regarding timing of diversification and evolution of pollination syndromes. – Ann. Bot. London **110**(1): 71–90.

Kitsaki C. K., Zygouraki S., Ziobora M. & Kintzios S. (2004): In vitro germination, protocorm formation and plantlet development of mature versus immature seeds from several *Ophrys* species (Orchidaceae). – Plant Cell Rep. **23**: 284–290. doi: 10.1007/s00299-004-0841-8.

LaCroix I. F. & LaCroix E. (1997): African orchids in the wild and in cultivation. – Portland, Oregon: Timber Press.

Malmgren S. (1996): Orchid propagation. Theory and practice. – In: Allen C. [ed.]: North American native terrestrial orchids. Propagation and production. – North Amer. Native Terrestrial Orchid Conf. Proc., Germantown, Md.: 63–71.

Martínez-García J. F., Virgós-Soler A. & Prat S. (2002): Control of photoperiod-regulated tuberization in potato by the *Arabidopsis* flowering-time gene *CONSTANS*. – PNAS **99**(23): 15211–15216.

Miyoshi K. & Mii M. (1995): Enhancement of seed germination and protocorm formation in *Calanthe discolor* (Orchideaceae) by NaOCl and polyphenol treatments. – Pl. Tiss. Cult. Lett. **12**(3): 267–272.

Mohamed-Yasseen Y., Splittstoesser W. E. & Litz R. E. (1994): In vitro shoot proliferation and production of sets from garlic and shallot. – Plant Cell Tissue Organ. Cult. **36**: 243–247.

Mohanty P., Paul S., Das M. C., Kumaria S. & Tandon P. (2012): A simple and efficient protocol for the mass propagation of *Cymbidium mastersii*: an ornamental orchid of Northeast India. – AoB Plants **2012:** pls023.

Murashige T. & Skoog F. (1962): A revised medium for rapid growth and bio-assays with tobacco tissue cultures. – Physiol. Pl.: **15**(3): 473–497.

Myers N., Mittermeier R., Mittermeier C., da Fonseca G. & Kent J. (2000): Biodiversity hotspots for conservation priorities. – Nature **403**(6772): 853–858.

Pridgeon A. M. (2001): Genera Orchidacearum: Orchidoideae 1: 313. – Oxford: University Press.

Puchooa D. (2004): Comparison of different culture media for the *in vitro* culture of *Dendrobium* (Orchidaceae). – Int. J. Agric. Biol. **6**(5): 884–888.

Szendrák E. (1997): Asymbiotic in vitro seed germination, micropropagation and scanning electron microscopy of several temperate terrestrial orchids (Orchidaceae). – Ph.D. Thesis, University of Nebraska, Lincoln.

Teixeria J. B, Sondahl M. R. & Kirby E. G. (1994): Somatic embryogenesis from immature inflorescences of oil palm. – Pl. Cell Rep. **13**(5): 247–250.

Thomas T. D. (2008): The role of activated charcoal in plant tissue culture. – Biotechnol. Advances **26**(6): 618–631.

Thompson D.I., Edwards T. & Van Staden J. (2003): Assessing a newly formulated dual-phase technique in orchid seed culture: conserving the South African *Disa in vitro.* – S. African J. Bot. **69**(2): 245.

Thompson D.I, Edwards T.J. & Van Staden J. (2006): A novel dual-phase culture medium promotes germination and seedling establishment from immature embryos in South African *Disa* (Orchidaceae) species. – Pl. Growth Regulat. **53**(3): 163–171.

Van der Cingel N.A. (2001): An atlas of orchid pollination: 133. – Boca Raton: CRC Press.

Van Waes J.M (1987): Effect of activated charcoal on *in vitro* propagation of western European orchids. – Acta Hort. **212**(21): 131–138.

Vogel S. (1954). Blütenbiologische Typen als Elemente der Sippengliederung. – Bot. Stud. (Jena) **1**: 1–338.

Waterman P.G & Mole S. (1994): Methods in ecology: analysis of phenolic plant metabolites. – Oxford, UK: Blackwell Scientific Publications.

Whigham D.F., McCormick M.K. & O'Neill J.P. (2008): Specialized seedling strategies II: orchids, bromeliads, carnivorous plants and parasites. – In: Leck M.A., Parker V.T. & Simpson R.L. [eds]: Seedling ecology and evolution **4**(1): 85. – Cambridge: University Press.

Addresses of the authors:

Maksim I. Antipin (author for correspondence)
Moscow State University
Biological faculty, Botanical Garden
Leninskiye Gory 1 (12)
119234 Moscow
Russian Federation

Elena A. Labunskaya
Vladimir V. Choob
Moscow State University
Biological faculty
Plant Physiology Department
Leninskiye Gory 1 (12)
119234 Moscow
Russian Federation