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Acclimatization of terrestrial orchid *Bletilla striata* Rchb.f. (Orchidaceae) propagated under *in vitro* conditions

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ABSTRACT

Bletilla striata is a terrestrial sympodial orchid. Substrates used for outdoor growing, differing in the mixture of added components and nutrients, were chosen for acclimatization of the asymbiotic propagated plants. A total of 651 *Bletilla striata* orchids were planted in 3 commercial substrates: ″Tonsubstrat″ (Ton), ″Baltisches substrat″ (Baltski) and ″Royal-Garden″ (Royal). Prior to acclimatization, the plants were 2.5 cm on average, with at least 2 leaves and 2 - 3 cm long root or roots. Fewest plants (3.1%) died in Ton substrate, 3.2% in Baltski substrate and 5.0% in Royal substrate. There were no statistically significant differences among substrates (p = 0.558) in the percentage of plants that died. Substrates used in combination with the chosen phenophase and established conditions were suitable for acclimatization of *Bletilla striata* orchids, whereby 95-97% of plants successfully adapted from heterotrophic to autotrophic conditions in a very short period of two months. The basic conditions for success are that plants are large and vital enough prior to acclimatization, that the substrate is appropriate and that appropriate conditions of relative humidity, temperature, light and ventilation without major fluctuations of these factors are ensured during acclimatization.

Key words: ornamental orchid, *Bletilla striata*, tissue culture, acclimatization, substrate, growth, development

IZVLEČEK

AKLIMATIZACIJA TERESTIČNE ORHIDEJE *Bletilla striata* Rchb.f. (Orchidaceae) RAZMNOŽENE V *in vitro* RAZMERAH

Bletilla striata je simpodialno razraščajoča se, v tleh rastoča orhideja. Za aklimatizacijo asimbiotsko razmnoženih rastlin smo izbrali substrate, ki se uporabljajo za gojenje rastlin na prostem ter se razlikujejo glede mešanice dodanih komponent in hranil. Skupno je bilo posajenih 651 orhidej v 3 komercialne substrate, ″Tonsubstrat″ (Ton), ″Baltisches substrat″ (Baltski) in ″Royal-Garden″ (Royal). Pred aklimatizacijo so imele rastline v povprečju 2,5 cm velik nadzemni del z vsaj dvema listoma in 2 do 3 cm dolgo korenino oz. korenine. Najmanj (3,1%) rastlin je propadlo v postopku aklimatizacije v Ton substratu, 3,2% v Baltskem substratu in največ 5,0% v Royal substratu. Med substrati ni bilo statistično značilnih razlik (p = 0,558) v odstotku propadlih rastlin med aklimatizacijo. Uporabljeni substrati v kombinaciji z izbrano fenofazo in vzpostavljene razmere so zelo primerni za aklimatizacijo te orhideje saj se je 95 do 97% rastlin uspešno prilagodilo iz heterotrofnih na avtotrofne razmere in to v zelo kratkem obdobju dveh mesecev. Osnovni pogoj uspeha je, da so *in vitro* rastline pred aklimatizacijo dovolj velike in vitalne, primeren substrat ter ustrezne razmere, vlaga, temperatura, svetloba in kroženje zraka, brez večjih nihanj v obdobju aklimatizacije.

Ključne besede: okrasna orhideja, *Bletilla striata*, tkivna kultura, aklimatizacija, substrat, rast, razvoj

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1 INTRODUCTION

Bletilla striata Rchb.f. (Orchidaceae) is a sympodial, terrestrial orchid originating from China and Japan. It has short rhizomes that develop corm-like pseudobulbs at ground level. Each pseudobulb bears several lance-shaped pleated leaves and is of annual duration only. On established plants, almost every new growth shoot has a flower spike before leaves fully develop. Inflorescences are terminal racemes, with up to 12 bell-shaped magenta flowers, with a ruffled lip, 3 cm in diameter (Brickell, 1996).

Bletilla is very easy orchid to cultivate. It was the second tropical orchid to be cultivated in Europe in the 18th century and is still often labeled simply 'Hardy Orchid' or 'Chinese Ground Orchid'. It is semi-hardy and needs protection from severe frost. It is deciduous, dying back to ground level in autumn. The blooms usually last a few weeks if conditions are good (Strong, 2000).

Bletilla is used in both traditional Chinese and in modern medicine. When employed in herbal remedies, the tuber is peeled and dried in the sun, then cut into slices or ground into a powder (Yeung, 1985). Among modern applications, *Bletilla* is often used in antibacterial, anti-inflammatory, anti-phlogistic, demulcent, pectoral, skin, styptic and vulnerary treatment and because of its astringent properties also to stop bleeding, heal wounds, reduce swelling and promote tissue regeneration (Singh and Duggal, 2009). Recent studies indicate that *Bletilla* can have an important role in the treatment of liver tumor (Qian et al., 2003). Another study (Diaoa et al., 2008) claims that *Bletilla striata* polysaccharide enhances the wound healing mechanism with an influence on macrophages.

The ultimate success of micropropagation on a commercial scale depends on the ability to transfer plants out of culture on a large scale, at low cost and with high survival rates (Chandra et al., 2010). Environmental conditions for *ex vitro* growth are quite different from those used for *in vitro* cultivation (Kozai et al., 1997). The acclimatization of *in vitro* plants is the last phase of micropropagation and is essential for the survival and successful establishment of plantlets. In other words, the survival percentage is determined by the hardening of the plantlets (Deb and Imchen, 2010). The conditions during *in vitro* culture result in the formation of plantlets of abnormal morphology, anatomy and physiology. After *ex vitro* transfer, plantlets endure shock because of sudden changes in environmental conditions. They need a period of acclimatization to correct the abnormalities (Pospišilova et al., 1999).

It is well documented that *in vitro* grown plantlets exhibit a low capacity for inorganic carbon assimilation because of their heterotrophic metabolism (Premkumar et al., 2001). The use of air-tight vessels in order to prevent contamination in tissue culture decreases air turbulence and limits the inflow of CO_2. The culture conditions also have very high air humidity and low irradiance, and the cultivation media are supplemented with saccharides (sucrose, glucose) as carbon and energy sources (Pospišilova et al., 1999). Under standard tissue culture conditions, the relative humidity is usually greater than 95%. *In vitro* leaves may not develop a waxy cuticle and functional stomata to the same extent as found in *ex vitro* plants (Seelye et al., 2003). Acclimatization of regenerates overcomes this threat by gradual lowering the air humidity (Bolar et al., 1998). Ventilation using loosely fitting closures or vents reduces the relative humidity, which leads to increases in plant transpiration and the development of functional stomata for controlling plant water loss (Seelye et al., 2003). During the acclimatization process, seedlings must overcome the critical phase when the heterotrophic behavior of the *in vitro* plants is shifted to autotrophic functioning.

The aim of our work was to optimize the acclimatization of *Bletilla striata* orchids as the final stage of successful micropropagation. We tested three different substrates and used mini-greenhouses for the procedure.

2 MATERIAL AND METHODS

2.1 Plant material

A *Bletilla striata* plant has been growing for some years outdoors in a garden in Ljubljana. Flowers were pollinated in summer, seed capsules were collected in October when fully mature and were stored in a refrigerator at 4 °C until use.

2.2 *In vitro* and *in vivo* propagation

Seeds were surface sterilized using dichloroisocyanuric acid (Sigma-Aldrich, St. Loius, MO, USA) in a 1.6% solution with Tween 20 (Sigma-Aldrich) added as surfactant. Seeds were then inoculated on commercial media Sigma P1056. Seedlings of a size of 1 to 2 cm, with second leaf indicated and at least one root, were transferred to sub-cultivation media consisting of macro elements of B5 media (Gamborg et al., 1968), micro elements of MS media (Murashige in Skoog, 1962) and other components, except banana powder, summarized from Hinnen et al. (1989). Plants 2 to 3 cm large with at least 2 leaves and 2 roots from the sub-cultivation media were randomly planted in 3 different substrates. Prior to transplantation, media was carefully cleaned or washed from the roots with distilled water to prevent pathogenic microorganisms developing. The cleaned plants were left in distilled water for protection against desiccation. They were then planted in substrate and immediately covered with a mini-greenhouse cover (Figure 1A, B and C). During acclimatization, the plants were watered with distilled water to prevent calcium carbonate deposition on plants, which could block stomata on leaves.

2.3 Substrate

Three commercial organic substrates were used in the acclimatization experiment: ″Tonsubstrat″, ″Baltisches substrat″ and ″Royal-Garden″. They are used for outdoor growing and are poor to middle-rich with nutrients

In our experiment, ″Tonsubstrat″ was labeled ″Ton″ and is a product of Klasmann-Deilmann GmbH, Germany. It consists of a mixture of poorly to medium decomposed white peat and very decomposed black peat and clay grains. The electrical conductivity of the substrate is 40 mS/m (+/- 25 %), pH value is 5.5 - 6.5 and the amount of added fertilizer is 1.5 kg/m^3 NPK 14:16:18.

″Baltisches substrat″ was labeled ″Baltski″ and is of Baltic origin, producer Hawita-Grupe GmbH, Germany. It consists of a mixture of clay grains, white peat, bark, humus, perlite and other components which are not specified. The pH value and salt content varies.

″Royal-Garden″ was labeled ″Royal″ and is a product of Humko Bled, Slovenia. It consists of a mixture of siliceous fine sand, vermiculite and clay. The pH value is 6, soluble nutrient content 180 - 350 mg/l N, 200 - 400 mg/l P_2O in 200 - 450 mg/l K_2O.

After random planting of seedlings in substrate, the process of acclimatization, i.e., adaptation to autotrophic metabolism started.

2.4 Growing place and acclimatization procedure

Orchids were acclimatized in plastic mini-greenhouse-like seed trays, consisting of two parts. The bottom part was dark green, made from more flexible plastic, while top part (cover) was harder and transparent. There were two openings on the cover, i.e., vents for ventilation of the growing area. The size of the bottom part was 36 × 22 × 6 cm and the size of cover 36 × 22 × 12 cm (Figure 1C).

After planting, the plants were moderately watered with distilled water. The substrate should not be too moist, because the plants have unhardened (thin and tender) cell walls in the hypocotyl part and can quickly become infected and die. We therefore placed two 50 ml beakers with water in each mini-greenhouse to establish a high relative humidity as soon as possible. The mini-greenhouses were placed in a shaded part of a greenhouse. After one week, the beakers with water were removed. The vents on the covers of the mini-greenhouses stayed closed for two weeks (Figure 1C) but the growing area was ventilated by removing the covers for a few minutes every day and then re-closed. The vents on the covers were gradually opened in the third week. In the fourth week, the covers were gradually lifted and, at the end of the week, completely removed. The orchids in the opened mini-greenhouses were watered at least once a week or as required depending on the moistness of the substrate.

2.5 Evaluation of data

The number of surviving and dead plants was monitored every two weeks. The first data were collected after the 2nd week of acclimatization, the second data collection was after the 5th week and the third in the 8th week, when the covers of the mini-greenhouses were removed and the plants were exposed to greenhouse conditions for two weeks. Data were processed using a logistic regression model.

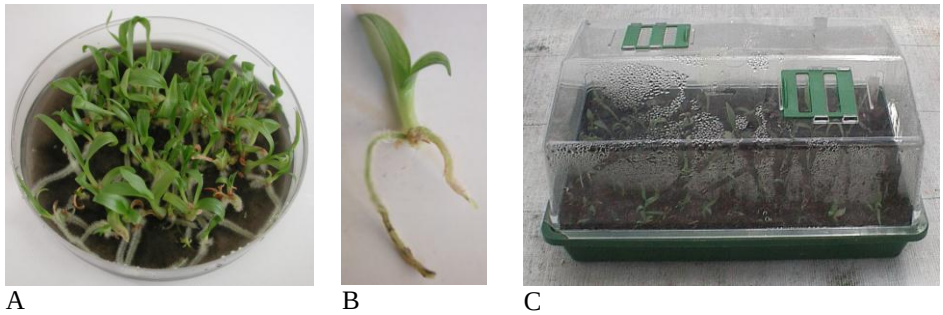
3 RESULTS AND DISCUSION

A total of 651 *Bletilla striata* orchids were planted in three commercial substrates, Ton, Baltski and Royal. They were asymbiotically propagated and grown *in vitro* until acclimatization (Figure 1A and B). All plants were subject to the same growth conditions except for the substrate. Mini-greenhouses were used for easier maintenance of conditions. Very high relative humidity was maintained in the growing area of the mini-greenhouses at the beginning, from 95-100%, which was later gradually reduced (Figure 1C). It is important for the substrate to maintain aeration in spite of the high relative humidity in the growing area. It is possible to establish a high relative humidity in mini-greenhouses quickly and successfully, which is very important for heterotrophic plant survival. Gordon (1991) considered five factors to be very important and they must be provided and controlled in tropical orchid acclimatization. In addition to humidity, these are substrate, temperature, light and air circulation. It is most important to maintain permanent conditions, i.e., to avoid stress.

Bletilla striata is a sympodial ground growing orchid, so we chose substrates that are used for growing outdoor plants and differ in the mixture of added components and nutrients.

No plants died in the first two weeks of acclimatization. During that period, the plants grew at high relative humidity, which varied from 95 to 100%, except during daily ventilation (Figure 1C). Such humidity was maintained at the beginning with water in beakers and, during the second week, by watering with distilled water only, in order not to block the stomata on leaves. Conditions during that period were very similar to *in vitro* conditions, except for the type and concentration of available nutrients and the light, which was suitable for photosynthesis.

In the third week, the vents on the covers of the mini-greenhouses were gradually opened and the relative humidity in the growing area was lowered. That influenced the function of the stomata and the formation and hardening of a wax cuticle on the leaves and stems.



A B C

Figure 1: Acclimatization of *Bletilla striata*: A - *in vitro* cultivated plants before acclimatization; B - media removed from roots before planting in substrate; C - acclimatization in mini-greenhouse.

The highest number of plants died during the 5th week of acclimatization, when the covers of the mini-greenhouses were totally removed and the plants were directly exposed to conditions in a greenhouse. The highest percentage of plants died during the fifth week was 2.2%, in Ton substrate. Percentage of plants died in Baltski substrate and Royal substrate was 1.8% and 1.1%, respectively (Table 1). Over the course of 5 weeks of acclimatization, the plants hardened and established stomata function and, consequently, transpiration. They did not therefore wilt after removal of the covers, although a few plants died during the next phase.

During the 8th week of acclimatization, the plants had already been exposed to greenhouse conditions for two weeks and dying was reduced, except in Royal substrate. Fewest plants (0.9%) died in Ton substrate, 1.4% in Baltski substrate but dying increased in Royal substrate to 3.9% of plants (Table 1). By that time, the plants had adapted from heterotrophic to autotrophic conditions and acclimatization was completed.

Table 1: Number of planted and dead plants of *Bletilla striata* during the acclimatization procedure and the results of the logistic regression model.

Substrate	**Number of planted plants**	**Number and percentage of dead plants**				**Total**	**Logistic model estimates**	
		5th week		**8th week**			**% of died**	**95% Conf. Int.**
Ton	198	5	2.2%	1	0.9%	6	3.1	1.2-6.4
Baltski	223	4	1.8%	3	1.4%	7	3.2	1.3-6.4
Royal	230	2	1.1%	9	3.9%	11	5.0	2.6-8.8

The acclimatization procedure was very successful in all substrates, 95-97% of plants survived and the number of dead plants was insignificant. There were no statistically significant differences among substrates (p = 0.558) in the percentage of dead plants (Table 1).

The acclimatized plants developed new leaves and roots, some of them also a pseudobulb and new shoots (Figure 2A, B and C).

A B C

Figure 2: *Bletilla striata* plants after acclimatization: A - plant with new leaves and roots after acclimatization; B plant with a new shoot after acclimatization; C - plant with pseudobulbs and a new shoot after acclimatization.

It is also important for plants to be exposed to light during acclimatization, which enables them to establish the process of photosynthesis. Other important conditions are an appropriate temperature without major fluctuations or air circulation. This was achieved by planting the plants in mini-greenhouses at the end of March, when the day is longer, and they were then placed in a greenhouse. When the period of hot weather started, the mini-greenhouses were moved to a shaded part of the greenhouse. Every day during the first two weeks, when the mini-greenhouses were completely closed, we removed the covers for a few minutes and exchanged the air in the growing area. Gordon (1991) recommended artificial light, whereby it is easier to control the length and intensity of illumination. Artificial light avoids seasonal differences or longer periods of cloudy weather and, in addition, partly solves the heating problem.

In addition to the listed factors and appropriate substrate, the size and developmental stage and vitality of *in vitro* cultivated plants are very important. The orchids included in the experiment were an average size of 2.5 cm, with at least 2 leaves and 2 - 3 cm long root or roots (Figure 1A and B). Croezen (2002) reported that the appropriate size for orchid acclimatization is when their leaves are at least 5 cm, while Park et al. (2003) recommended a shoot size of 3 - 4 cm with two leaves and 3 - 4 roots. Nayak et al. (1997) recommended for the monopodial orchid *Acampe praemorsa* (Roxb.) Blatt. & McCann a shoot with an average of 4.5 roots, 3.7 cm long. Chang and Chang (1998) state that the appropriate size of regenerants for the sympodial orchid *Cymbidium ensifolium* (L.) Swartz is 5 cm. In other literature, there are different data depending on the genus or species of orchid.

Our plants were smaller and with fewer roots than stated in the aforementioned literature. No data are available in the literature for *Bletilla striata* orchid, so we decided the appropriate size and phenophase ourselves, based on when the plants have at least the minimum of nutrients stored in the leaves and roots required for the acclimatization process. By using the minimum plant size possible to acclimatize we shortened the period of *in vitro* cultivation, which is very important for mass market production.

The percentage of acclimatized plants was high (95 - 97%) and was obtained in two months, which is very fast according to the literature. All factors were not optimal, as recommended for other genera and species of tropical orchids. Chen et al. (2002) reported 90% successful acclimatization for the sympodial orchid *Epidendrum radicans* Lindl. Chen and Chang (2000) reported 100% success for the sympodial orchid *Oncidium*. Murthy and Pyati (2001) reported 84% acclimatization after 3 months for the monopodial orchid *Aerides maculosum* Lindl.

4 CONCLUSIONS

The use of mini-greenhouses was very handy for the acclimatization of a smaller to medium number of plants. It allows optimal relative humidity, temperature and ventilation to be maintained easily without much investment. Some processes that were included in acclimatization - appropriate ventilation of the growing area, temperature, no exposure to direct sunlight and the provision of shade significantly affected plant survival. We also observed that acclimatization success depended on the interaction of the aforementioned factors, on avoiding major fluctuations of these factors and of suitable phenophase and vitality of the *in vitro* cultivated plants. Plants with 2.5 cm average size with at least 2 leaves and 2 - 3 cm long root or roots were

very suitable. Substrates used in combination with the chosen phenophase and established conditions were suitable for the acclimatization of a smaller number of *Bletilla striata* orchids, whereby 95-97% of plants successfully adapted from heterotrophic to autotrophic conditions in a very short period of two months.

It can be concluded on the basis of the presented data that the fundamental conditions for success are size, vitality and an appropriate stock of nutrients in the leaves and roots of *in vitro* propagated plants, as well as appropriate measures during the acclimatization period.

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6 REFERENCES

Bolar, J.P., Norelli, J.L., Aldwinckle H.S., Hanke V. 1998. An efficient method for rooting and acclimation of micropropagated apple cultivars. HortScience 37: 1251-1252.

Bown, D. 1995. Encyclopedia of Herbs and Their Uses. London, Dorling Kindersley, 424 p.

Brickell, C. 1996. The RHS A-Z Encyclopedia of Garden Plants. London, Dorling Kindersley, 1136 p.

Chandra, S., Bandopadhyay, R., Kumar, V., Chandra, R. 2010. Acclimatization of tissue cultured plantlets: from laboratory to land. Biotechnol. Lett. 32: 1199-1205.

Chang C., Chang W. C. 1998. Plant regeneration from callus culture of *Cymbidium ensifolium* var. misericors. Plant Cell Reports 17: 251-255.

Chen J.T., Chang W.C. 2000. Plant regeneration via embryo and shoot but formation from flower-stalk explants of *Oncidium* sweet sugar. Plant Cell, Tissue and Organ Culture 62: 95-100.

Chen L.R., Chen J.T., Chang W.C. 2002. Efficient production of protocorm-like bodies and plant regeneration from flower stalk explants of the sympodial orchid *Epidendrum radicans*. In Vitro Cellular & Developmental Biology-Plant 38: 441-445.

Croezen P. 2002. In vitro orchid cultivation. OrchidMania Inc. http://www.orchids.org/conservation/inVitro.html

Deb, C.R., Imchen, T. 2010. An efficient *in vitro* hardening technique of tissue culture raised plants. Biotechnology 9: 79-83.

Diaoa, H., Lia, X., Chena, J., Luoa, Y., Chena, X., Donga, L., Wanga, C., Zhanga, C., Zhanga, J. 2008. *Bletilla striata* Polysaccharide Stimulates Inducible Nitric Oxide Synthase and Proinflammatory Cytokine Expression in Macrophages. Journal of Bioscience and Bioengineering 105: 85-89.

Feng, G., Kramann, B., Zheng, C., Zhou, R. 1996. Comparative study on the long-term effect of permanent embolization of hepatic artery with bletilla striata in patients with primary liver cancer. J of Tongji Med Univ. 16:111-116.

Gamborg O. L., Miller R. A., Ojima K. 1968. Nutrient requirementes of suspension cultures of soybean root cells. Experimental Cell Researsh 50: 151-158.

Gordon, B. 1991. Orchid Seedling Care (with special emphasis on water quality). Running Springs, Laid-Back Publications, 163 p.

Hinnen M.G.J., Pierik R.L.M., Bronsema F.B.F. 1989. The influence of macronutrients and Some Other Factors on Growth of *Phalaenopsis* hybrid seedlings *in vitro*. Scientia Horticulturae 41: 105-116.

Kozai, T., Kubota, C., Jeong, B.R., 1997. Environmental control for the large-scale production of plants through in vitro techniques. Plant Cell, Tissue and Organ Culture 51: 49-56.

Leroy-Terquem, G., Parisot, J. 1993. Orchids Care and cultivation. London, Cassel & Co., 200 p.

Murashige T., Skoog F. 1962. A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiologia Plantarum 15: 473-497.

Murthy H.N., Pyati A.N. 2001. Micropropagation of Aerides maculosum Lindl. (Orhidaceae). In Vitro Cellular & Developmental Biology-Plant 37: 223-226.

Nayak N.R., Patnaik S., Rath S.P. 1997. Direct shoot regeneration from foliar explants of an epiphytic orchid, *Acampe praemorsa* (Roxb.) Blatter and McCann. Plant Cell Reports 16: 583-586.

Park S.Y., Murthy H.N., Paek K.Y. 2003. Protocorm-like body induction and subsequent plant regeneration from root tip cultures of Doritaenopsis. Plant Science 164: 919-923.

Pospišilova, J., Ticha, I., Kadleček, P., Haisel, D., Plzakova, Š. 1999. Acclimatization of micropropagated plants to ex-vitro conditions. Biol. Plant. 42: 481-497.

Premkumar, A., Mercado, J.A., Quesada, M.A. 2001. Effects of *in vitro* tissue culture conditions and acclimatization on the contents of Rubisco, leaf soluble proteins, photosynthetic pigments, and C/N ratio. J. Plant Physiol. 158: 835–840.

Qian, J., Vossoughi, D., Woitaschek, D., Oppermann E., Bechstein W.O., Li W.-Y., Feng, G.-S., Vogl, T. 2003. Combined transarterial chemoembolization and arterial

administration of *Bletilla striata* in treatment of liver tumor in rats. World J Gastroenterol. 9: 2676-2680.

Seelye, J.F., Burge, G.K., Morgan, E.R. 2003. Acclimatizing Tissue Culture Plants: Reducing the Shock. Combined Proceedings International Plant Propagators' Society 53: 85-90.

Singh, A., Duggal, S. 2009. Medicinal Orchids - An Overview. Ethnobotanical Leaflets 13: 399-412.

Strong, G. 2000. A Gardener's Guide to Orchids and Bromeliads. London, Murdoch books, 96 p.

Yeung, H.C. 1985. Handbook of Chinese Herbs. Institute of Chinese Medicine, 673 p.