Journal of Genetic Engineering and Biotechnology (2014) **12**, 71–74

Academy of Scientific Research & Technology and National Research Center, Egypt

Journal of Genetic Engineering and Biotechnology

www.elsevier.com/locate/jgeb

ARTICLE

Successful storage of protocorm-like bodies of hybrid *Cymbidium* (Orchidaceae) under low temperature conditions

Jaime A. Teixeira da Silva *

Faculty of Agriculture and Graduate School of Agriculture, Kagawa University, Miki-cho, Kagawa 761-0795, Japan
Current address: Miki cho Post Office, Ikenobe 3011-2, P.O. Box 7, Kagawa-Ken 761-0799, Japan

Received 30 August 2013; revised 27 November 2013; accepted 7 December 2013
Available online 2 January 2014

KEYWORDS

Cold;
Cryopreservation;
Low-temperature storage;
PLB;
Teixeira *Cymbidium* (TC) medium

Abstract Low temperatures result in lower metabolic cellular activity, thus slowing down cell division and growth. This is advantageous where a plant scientist might seek to store important germplasm without the risks associated with low temperature storage. In this study, two cold temperatures above freezing, namely 4 and 10 °C, were tested to assess for how long PLBs could be preserved without a significant loss in regeneration ability (i.e., the ability to form *neo*-PLBs). Control treatments were cultured at 25 °C on Teixeira *Cymbidium* (TC) medium at a 16-h photoperiod at a photosynthetic photon flux density (PPFD) of 45 μmol m^{-2} s^{-1}. For the cold treatments, each was replicated in the dark and at low light intensity (12-h photoperiod and a PPFD of 10 μmol m^{-2} s^{-1}). All cultures were sub-cultured six times onto fresh medium every 60 days, for approximately 1 year. On the 7th subculture, all *neo*-PLBs were prepared uniformly and replated onto standard TC medium under light conditions described above for the control. 45 days after the 7th subculture and just before subcultures 1–6, the number of *neo*-PLBs per half-PLB was measured. The number of *neo*-PLBs that formed under different treatments depended strongly on the temperature and light conditions with most *neo*-PLBs forming under control conditions, although that number dropped significantly as the temperature was dropped to 10 °C and then even more to 4 °C, the same trend being observed when explants were cultured and subcultured under dim light, with organogenesis being more strongly negatively influenced in darkness. For all low-temperature treatments, as well as the dimmed light and darkness treatments, the number of *neo*-PLBs increased significantly when recultured, on the 7th subculture, onto control TC medium under control environmental conditions, almost as high as the control values. In contrast, the control values decreased, with significantly fewer *neo*-PLBs by the 7th subculture relative to the control, indicating that new PLBs should be induced from shoot cultures at least once a year to maintain their vitality.

* Tel.: +81 878988909.
E-mail address: jaimetex@yahoo.com

Peer review under responsibility of National Research Center, Egypt.

Production and hosting by Elsevier

1. Introduction

Cymbidium (Orchidaceae) is an economically important orchid whose development *in vitro* is now being well studied [8,20,21]. Protocorm-like bodies (PLBs) are the best clonal propagules as

1687-157X © 2013 Production and hosting by Elsevier B.V. on behalf of Academy of Scientific Research & Technology.
http://dx.doi.org/10.1016/j.jgeb.2013.12.001

they are somatic embryos [26] that can be multiplied through the induction of new PLBs or *neo*-PLBs and can thus be used as explants for synthetic seeds [17,19], for feed-batch culture and bioreactor studies (unpublished data) or for cryopreservation [22]. There is interest in mid-term (several weeks to months) and long-term (many months to several years) storage of *Cymbidium* germplasm, not only as a way to preserve genetic material and rare or endangered germplasm, but also – in a world of increasing energy costs – as a way to reduce the costs associated with tissue culture, specifically lighting, heating, chemicals and reagents, and man-power. Although hybrid *Cymbidium* PLBs can be cryopreserved for as long as a year, the regeneration ability clearly declines [22]. This can be improved by tweaking cryopreservation conditions, or by the use of an alternative method. One such alternative is low-temperature storage (LTS), the aim of this study.

Cha-um and Kirdmanee [2] list several ways in which *in vitro* plant cultures can be maintained for longer under minimal growth: physical factors, including LTS (while temperate material would allow for temperatures 2–10 °C, tropical material would most likely require 15–25 °C), low light intensity and short photoperiod, application of ethylene inhibitors, decreasing the size of the culture vessel (or increasing plant density per vessel); chemical factors, including minimal media and the manipulation of the concentration of plant growth regulators (reduction or removal) and osmotic potential. LTS is a way to preserve fresh produce (i.e., fruits, vegetables and cut flowers [2]), to induce bulbs to sprout through forcing (e.g., 0–10 °C terminates dormancy in *Paeonia lactiflora* [14]), to preserve seeds as reproductive propagules (e.g., Kew Gardens Millennium Seed Bank Project [10]), to preserve pollen (e.g., [12]) or to preserve vegetative tissues (e.g., strawberries, most LTS studies preserving shoot tips [4,5]). LTS, which could constitute a stress and thus induce cold shock proteins [15], results in changes in physiological, biochemical and genetic characteristics of the storage tissue ([2] and references therein). This study focuses on the latter objective for hybrid *Cymbidium* germplasm.

2. Results and discussion

The number of *neo*-PLBs that formed under different treatments depended strongly on the temperature and light conditions. Most *neo*-PLBs formed under control conditions, but that number dropped significantly as the temperature was dropped to 10 °C and then even more to 4 °C. The same trend was observed when explants were cultured and subcultured under dim light, with organogenesis being more strongly negatively influenced in darkness (Fig. 1). For all LTS treatments, as well as the dimmed light and darkness treatments, the

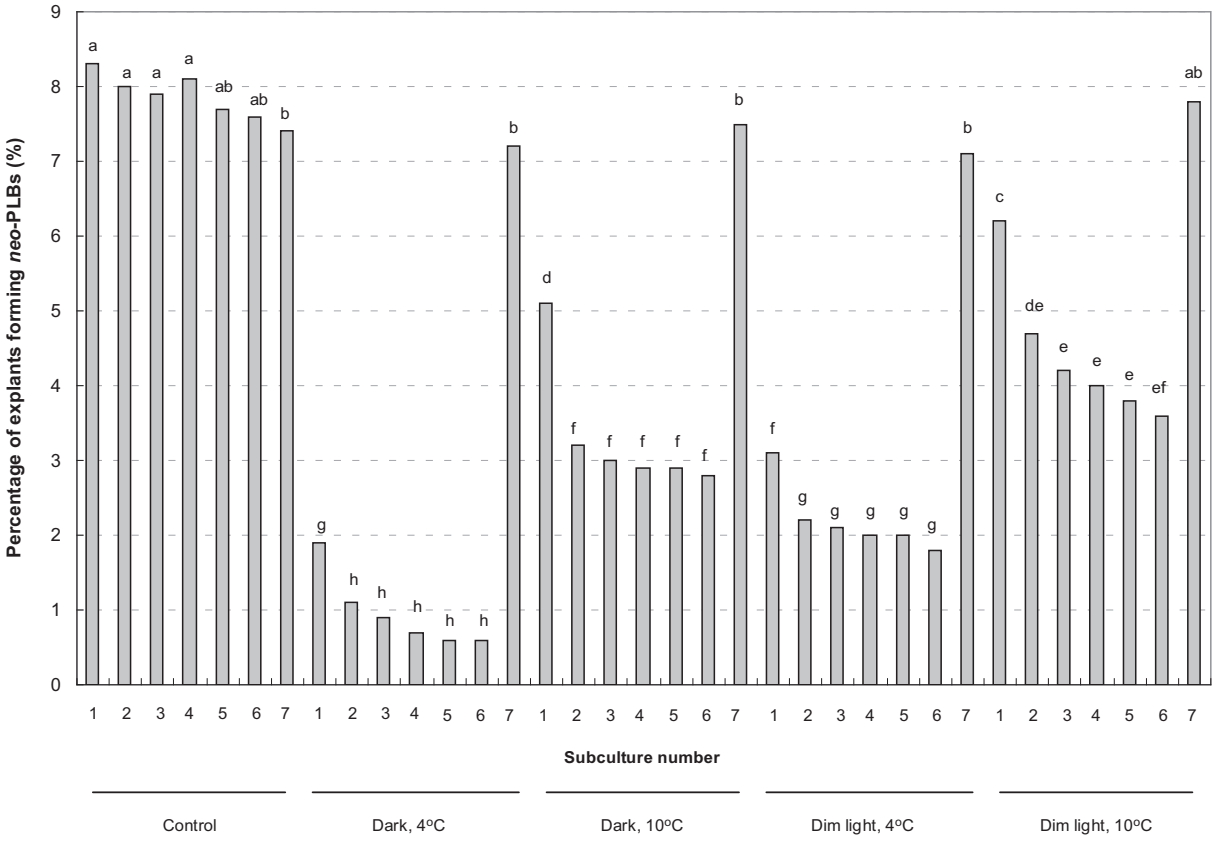

Figure 1 Development of *neo*-PLBs under control (25 °C, 16-h photoperiod, 45 μmol m^{-2} s^{-1}) (A), complete darkness (B), and dim light (12-h photoperiod, 10 μmol m^{-2} s^{-1}) conditions. B and C were performed under two low-temperature storage treatments, 4 °C and 10 °C. Each subculture 1–6 was performed after 60 days for all treatments and on the 7th subculture, half-PLBs prepared from *neo*-PLBs of all treatments were replated under control conditions. Mean values followed by the same letter across all treatments (including the control) are not significantly different based on DMRT ($P = 0.05$). $n = 90$.

number of *neo*-PLBs increased significantly when recultured, on the 7th subculture, onto control Teixeira *Cymbidium* (TC) medium [18,19] under control environmental conditions, almost as high as the control values (Fig. 1). Ironically, the control values tended to decrease, with productivity having decreased significantly by the 7th subculture relative to the control, suggesting that new PLBs should be induced from shoot cultures at least once a year to maintain their vitality. This indicates that even though LTS negatively influences *neo*-PLB formation (i.e., organogenesis) in hybrid *Cymbidium* up to the 6th subculture, a strong recovery of the vitality of PLBs is possible by the 7th subculture (Table 1, Fig. 1). Even though this trend decreases as the number of subcultures increases from 1 to 6, the subcultured half-PLBs, derived from the previous subculture of *neo*-PLBs, retain their organogenic potential for at least 1 year (approx 360 days), as observed by their performance in the 7th subculture, assessed by the number of neo-PLBs per half-PLB explant (Fig. 1), the percentage of explants forming *neo*-PLBs, and the fresh weight of PLB explants + *neo*-PLBs (Table 1). This is a very positive result and development, indicating that PLB germplasm can be stored under LTS for hybrid *Cymbidium*. The author suspects that the response will be different for different genotypes, and it would be useful to expand the treatments to test 15 °C, 20 °C and room temperature to see whether these conditions can improve the regenerative performance of this orchid in response to LTS.

This study proposes a medium-term storage option of hybrid *Cymbidium* germplasm. Short-term storage options for this hybrid already exist (e.g. [21,23,25,27]), while a long-term cryopreservation protocol has also been developed [22]. LTs can induce a number of negative physiological responses. For example, chilling of LTS cabbage induced water deficit [16]. The plant plasmalemma, ultrastructural molecules and intracellular pH are damaged by cold-induced injury (e.g., [1,2] and references therein). This in turn can lead to electrolyte leakage, pigment degradation and diminished photosynthesis (e.g., [2,13] and references therein). Reactive oxygen species, in response to cold stress, evolve, causing cell death (e.g., [6,9]). Cold acclimation, the treatment of plants or plant tissues

Table 1 Effect of subculture of hybrid *Cymbidium* Twilight Moon 'Day Light' half-PLBs on *neo*-PLB formation on TC medium for 7* subcultures under different light vs temperature conditions.

Medium composition	Subculture (each for 60 days)	Explants forming *neo*-PLBs (%)	Fresh weight (mg) of PLB explant + *neo*-PLBs
Control	1	100 a	526 a
25 °C	2	98 a	518 a
16-h photoperiod	3	100 a	531 a
45 μmol m^{-2} s^{-1}	4	100 a	514 a
	5	100 a	511 ab
	6	96 a	509 ab
	7	93 b	493 b
Darkness 4 °C	1	51 f	241 f
	2	29 g	136 i
	3	27 g	147 hi
	4	23 gh	139 i
	5	26 h	141 hi
	6	20 h	128 i
	7	64 e	373 de
Darkness 10 °C	1	72 d	418 d
	2	64 e	399 d
	3	66 de	391 d
	4	59 e	364 e
	5	61 e	352 e
	6	56 ef	341 e
	7	71 d	455 c
4 °C	1	76 cd	383 de
12-h photoperiod	2	63 e	206 g
10 μmol m^{-2} s^{-1}	3	61 e	203 g
	4	54 f	187 g
	5	52 f	179 gh
	6	47 f	164 h
	7	78 cd	409 d
10 °C	1	84 c	432 cd
12-h photoperiod	2	77 cd	404 d
10 μmol m^{-2} s^{-1}	3	65 de	401 d
	4	68 de	397 d
	5	64 e	381 de
	6	61 e	374 de
	7	83 c	489 b

Mean values followed by the same letter in the same column are not significantly different based on DMRT ($P = 0.05$). $n = 90$ (9 (3 × 3 blocks) Petri dishes × 10 for each treatment) for each subculture. *In the 7th subculture, all treatments were subcultured on control medium under control conditions. *Abbreviations:* PLB, protocorm-like body; TC, Teixeira *Cymbidium* medium (Teixeira da Silva 2012a), includes 0.1 mg/l α-naphthaleneacetic acid (NAA) and 0.1 mg/l kinetin, 2 g/l tryptone and 20 g/l sucrose (see reference for modified micro- and macro-nutrients).

to semi-low temperatures or other physical or chemical treatments prior to LTS, can increase the survival of plants once exposed to LTS (e.g., [28]), through the production of antifreeze proteins or the induction of cold-shock proteins [7]. Cold temperature regulation also occurs at the genetic level, with upstream transcription factors and downstream cold-responsive genes being activated or repressed (e.g., [3]). Reduced light intensity, as used in the dim light and reduced PPFD treatments in this study, slow down photosynthetic activity [11], although morphologically and physiologically normal plants could be recovered in this study when all LTS-treated *neo*-PLBs were re-cultured onto control TC medium on the 7th subculture, i.e., even after approximately 360 days in cold storage.

3. Materials and methods

All chemicals and reagents were of the highest analytical grade available and were purchased from either Sigma–Aldrich (St. Louis, USA), Wako Chemical Co. (Osaka, Japan) or Nacalai Tesque (Kyoto, Japan), at the highest tissue-culture grade. PLBs of hybrid *Cymbidium* Twilight Moon 'Day Light' (Lovely Bunny 'Romeo' X Hiroshima Golden Cup 'Sunny Moon'; Bio-U, Japan) were induced and subcultured every two months on TC medium, employing all culture conditions and recommendations defined by Teixeira da Silva [18,22]. Two cold temperatures above freezing, namely 4 and 10 °C, were tested to assess for how long PLBs could be preserved without a significant loss in regeneration ability (i.e., the ability to form *neo*-PLBs). Control treatments were cultured at 25 °C on TC medium at a 16-h photoperiod at a photosynthetic photon flux density (PPFD) of 45 μmol m^{-2} s^{-1}. For the cold treatments, each was replicated in the dark and at low light intensity (12-h photoperiod and a PPFD of 10 μmol m^{-2} s^{-1}). All cultures were sub-cultured onto fresh medium every 60 days from the 1st to the 6th subculture (i.e., for approximately 1 year). On the 7th subculture, all neo-PLBs were prepared uniformly and replated onto standard TC medium under light conditions described above for the control. The number of *neo*-PLBs per half-PLB was measured 45 days after the 7th subculture and just before every other subculture from the 1st to the 6th subculture, based on Teixeira da Silva and Dobránszki [24]. Experiments were organized according to a randomized complete block design (RCBD) with three blocks of 10 replicates per treatment. All experiments were repeated in triplicate ($n = 30$, total sample size per treatment). Data were subjected to analysis of variance (ANOVA) with mean separation by Duncan's multiple range test (DMRT) using SAS® vers. 6.12 (SAS Institute, Cary, NC, USA). Significant differences between means were assumed at $P \leqslant 0.05$.

4. Conclusions

Cymbidium PLBs can be stored at low temperatures (4 °C and 10 °C) and reduced light intensity (PPFD of 10 μmol m^{-2} s^{-1}) for up to approximately one year (6 × 60-day subcultures). Although PLB growth and *neo*-PLB formation under low temperature are significantly inferior under low temperatures, these parameters can be significantly recovered when PLBs are recultured onto control medium under control temperature and light conditions in the 7th subculture.

Acknowledgement

The author thanks Prof. Michio Tanaka for research support.

References

[1] R. Aroca, G. Amodeo, S. Fernandez-Illescas, E.M. Herman, F. Chaumont, M.J. Chrispeels, Plant Physiol. 137 (2005) 341–353.
[2] S. Cha-um, C. Kirdmanee, Fruit Veg. Cereal Sci. Biotech. 1 (1) (2007) 13–25.
[3] V. Chinnusamy, J. Zhu, J.K. Zhu, Physiol. Plant. 126 (2006) 52–61.
[4] S.C. Debnath, J.A. Teixeira da Silva, Fruit Veg. Cereal Sci. Biotech. 1 (1) (2007) 1–12.
[5] F. Engelmann, In Vitro Cell. Dev. Biol. Plant 47 (2011) 5–16.
[6] I. Ensminger, F. Busch, N.P. Huner, Physiol. Plant. 126 (2006) 28–44.
[7] M. Griffith, M.W.F. Yaish, Trends Plant Sci. 9 (2004) 399–405.
[8] M.M. Hossain, R. Kant, P.T. Van, B. Winarto, S.-J. Zeng, J.A. Teixeira da Silva, Crit. Rev. Plant Sci. 32 (2013) 69–139.
[9] S.R. Kalberer, M. Wisniewski, R. Arora, Plant Sci. 171 (2006) 3–16.
[10] Kew Gardens Millennium Seed Bank Project (2013). www.kew.org/msbp (last accessed: 25 November, 2013).
[11] H.K. Lichtenthaler, S. Burkart, Bulgar. J. Plant Physiol. 25 (1999) 3–16.
[12] P. Martínez-Gómez, T.M. Gradziel, E. Ortega, F. Dicenta, Hort. Sci. 37 (2002) 691–692.
[13] J. Renaut, J.F. Hausman, M.E. Wisniewski, Physiol. Plant. 126 (2006) 97–109.
[14] Y.-H. Rhie, H.-H. Jung, K.-S. Kim, Hort. Environ. Biotechnol. 53 (2012) 277–282.
[15] K. Sasaki, R. Imai, Front. Plant Sci. 2 (2012). Article 116.
[16] F. Sato, H. Yoshioka, T. Fujiwara, H. Higashio, A. Uragami, S. Tokuda, Sci. Hort. 101 (2004) 349–357.
[17] S. Sharma, A. Shahzad, J.A. Teixeira da Silva, Biotech. Adv. 31 (2013) 186–207.
[18] J.A. Teixeira da Silva, J. Fruit Ornament. Plant Res. 20 (2012) 127–133.
[19] J.A. Teixeira da Silva, J. Fruit Ornament. Plant Res. 20 (2012) 135–146.
[20] J.A. Teixeira da Silva, Floricult. Ornament. Biotech. 7 (2013) 1–52.
[21] J.A. Teixeira da Silva, Plant Cell Tissue Organ Cult. 113 (2013) 149–161.
[22] J.A. Teixeira da Silva, In Vitro Cell. Dev. Biol. Plant 49 (2013c) 690–698.
[23] J.A. Teixeira da Silva, M.-T. Chan Sanjaya, M.-L. Chai, M. Tanaka, Sci. Hort. 109 (2006) 368–378.
[24] J.A. Teixeira da Silva, J. Dobránszki, Sci. Hort. 159 (2013) 59–66.
[25] J.A. Teixeira da Silva, N. Singh, M. Tanaka, Plant Cell Tissue Organ Cult. 84 (2006) 119–128.
[26] J.A. Teixeira da Silva, M. Tanaka, J. Plant Growth Reg. 25 (2006) 203–210.
[27] J.A. Teixeira da Silva, T. Yam, S. Fukai, N. Nayak, M. Tanaka, Prop. Ornament. Plant 5 (2005) 129–136.
[28] B. Zhou, Z. Guo, Z. Liu, Crop. Sci. 45 (2005) 599–605.