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Cryopreservation of Shoot Tips of *Dendrobium* Walter Oumae by Encapsulation/Dehydration

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ABSTRACT: *In vitro* shoot tips of Dendrobium Walter Oumae were cryopreserved using the encapsulation/dehydration technique. Shoot tips were encapsulated in calcium-alginate before preculture on modified Vacin and Went (1949) agar medium supplemented with 0.3, 0.5 and 0.7 M sucrose for 2 d at 25±2 °C, 37 μmol $m^{-2}s^{-1}$ for 16 h per d. Encapsulated shoot tips were then dehydrated by incubation in the sterile air flow of a laminar air-flow cabinet for 0-10 h and immediately plunged into liquid nitrogen (LN). After recovering from LN and rapid thawing in a waterbath (40±2 °C), the survival ratio and regrowth were measured by a 2,3,5-triphenyl tetrazolium chloride (TTC) assay and a regrowth culture test, respectively. The highest survival ratio (16.18 mg living cells/100 mg total cells) and regrowth (13.33%) were obtained from encapsulated shoot tips that were previously precultured on 0.3 M sucrose agar medium for 2 d and sufficiently dehydrated for 6-8 h before storing in LN. The regrowth of plantlets was measured from length of shoot tips, number of shoots and roots which did not show any significant difference when air-dehydration and LN were tested. Finally, the regrown shoot tips could develop directly into complete plantlets without protocorms formation and they had normal morphology.

KEYWORDS: cryopreservation, encapsulation/dehydration, *Dendrobium*, shoot tip, TTC assay.

INTRODUCTION

Orchids are one of the most important ornamental plants in Thailand. They exhibit an incredible range of diversity in size, shape and color of their flowers.[1] At present, many of them are endangered due to many causes, such as the environmental changes, deforestation, wild orchid trade and other factors.[2] Therefore, there is an urgent need for Thai orchid conservation.

Cryopreservation is an important tool for long-term storage of biological materials. It offers a safe and cost-effective option for long-term conservation of genetic resources in many plant species. At the temperature of liquid nitrogen (LN, -196°C), all the metabolic activities of cells are at a standstill. Thus, they can be preserved in such a state for a long period.[3] Many new cryopreservation techniques such as simple freezing, vitrification, encapsulation/ dehydration and encapsulation/vitrification have been reported for successful use for many cells, tissues and organs of plant species.[4, 5, 6] However, for successful cryopreservation, many factors are involved, such as starting materials, pretreatment conditions, cryoprocedures and post-thaw treatment.[7] Therefore, in order to accomplish successful cryopreservation for each species and cultivar, a separate study must be carried out.

The objectives of this study is to establish efficient techniques for orchid conservation by cryopreservation using shoot tips as explants. Cryopreservation provides an important practical approach to orchid cryopreservation when compared with *in vitro* culture. It is relatively inexpensive, requires less space and resources and reduces the problems associated with somaclonal variations.[3] Only a few reports have been published on cryopreservation of orchids using seeds, protocorms and shoot tips.[2, 8, 9, 10] In this study *Dendrobium* Walter Oumae, which is a popular commercial cut-flower orchid cultivar in Thailand is used as a model for *Dendrobium* shoot tip cryopreservation by using encapsulation/dehydration technique.

MATERIALS AND METHODS

Plant Materials

About two-inch-long young shoots of *Dendrobium* Walter Oumae were obtained from the saranhouse of the Institute of Science and Technology for Research

and Development, Salaya campus, Mahidol University. They were cleaned and surface-sterilized with 10% Clorox® + Tween-20® for 10 min, 5% Clorox® + Tween-20® for 5 min and finally, 1% Clorox® for 1 min. Then apical meristems (1-2 mm in length) and axillary buds (3-5 mm in length) were cut from shoots with aseptic technique and cultured in modified Vacin and Went, 1949 (VW) liquid medium under 110 rpm rotation, 25±2 °C and 37 μmol $m^{-2}s^{-1}$ for 24 h. After 3-4 months, protocorms were formed and then subcultured on modified VW agar medium supplemented with 10% (w/v) banana and 0.5 g/l activated charcoal for inducing of *in vitro* orchid plantlets. The microshoots were subcultured to fresh medium at 8-week intervals. The culture conditions for *in vitro D.* Walter Oumae throughout this study were maintained at 25±2 °C, under white fluorescent lamps Philips TLD 36W/54 at the intensity of 37 μmol$m^{-2}s^{-1}$ for 16 h per d.[2]

Apical shoot tips (1-3 mm in length), comprising the meristematic dome with one or two unfolded leaves, were excised under a stereo microscope from 3 to 5 cm long orchid plantlets.

Cryopreservation

After excision, shoot tips were cryopreserved by the encapsulation/dehydration technique of Sakai.[3] *In vitro* shoot tips were suspended in calcium-free modified VW liquid medium supplemented with 3% (w/v) sodium alginate plus 0.4 M sucrose. The shoot tips with suspension were dropped into 0.1 M $CaCl_2$ solution. After 40 min, each bead (4-5 mm in diameter) containing one meristem was recovered. Subsequently, encapsulated shoot tips were precultured on modified VW agar medium supplemented with 0.3, 0.5 and 0.7 M sucrose for 2 d under the standard culture conditions. Then encapsulated shoot tips were gradually

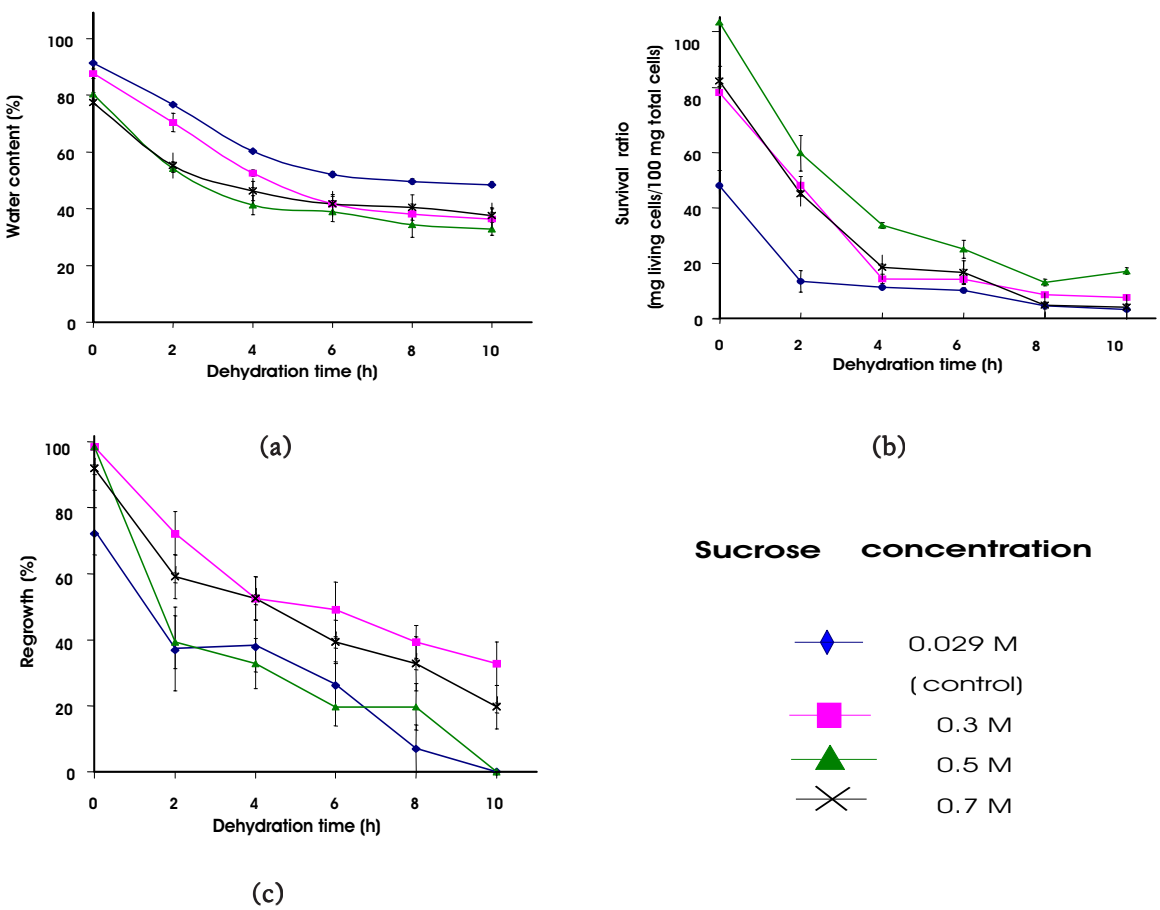

Fig 1. Effect of the duration of dehydration time (0-10 h) on water content (a), survival ratio (b) and regrowth (c) of encapsulated shoot tips, precultured on modified VW agar medium supplemented with 0.029 (control), 0.3, 0.5 and 0.7 M sucrose for 2 d. Vertical bars represent ± SD.

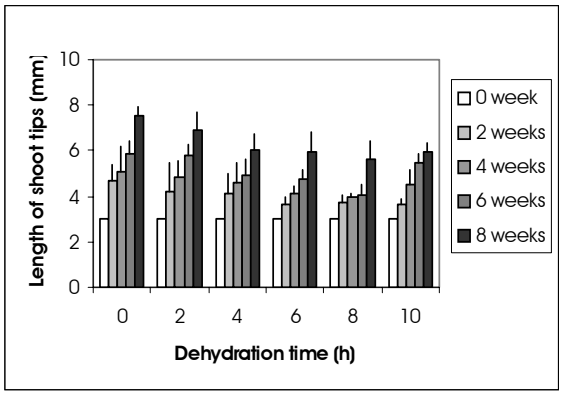

Fig 2. Effect of the duration of dehydration time on shoot tip regrowth. Shoot tips were precultured on modified VW agar medium supplemented with 0.3 M sucrose for 2 d before dehydration for 0-10 h without liquid nitrogen. Shoot tips were measured every 2 weeks after reculturing. Vertical bars represent ± SD.

dehydrated for 0-10 h, by exposed to sterile air-flow at 0.5 inches / water column from a laminar air-flow cabinet. Finally, the beads were placed into cryotubes and immediately plunged into LN. Samples were stored in LN for at least 1 d.

Each experiment was repeated 3 times and 10 and 15 shoot tips were used for survival ratio and regrowth evaluation, respectively.

Determination of Bead Water Content

Three independent samples of 10 beads were weighed at different times during the dehydration treatment and then placed in hot air oven (105 °C) for 24 h in order to weigh the dried explant.[12] The water content was expressed on a fresh weight (FW) basis.

Evaluation of Survival Ratio and Regrowth

Cryopreserved shoot tips were taken from LN tank and immediately thawed in a waterbath at 40±2 °C for 2 min. The shoot tips were then treated with 1.2 M sucrose in modified VW liquid medium (unloading solution) at 25±2 °C for 20 min, and cultured on modified VW agar medium under the culture condition.

Survival ratio was estimated by the 2, 3, 5- triphenyl tetrazolium chloride (TTC) reduction assay according to Ishikawa *et al*. (1995).[13] Regrowth ability of shoot tips was expressed as a percentage of total number of shoots forming normal shoots at the 4th week after culture.

Growth Measurement of Plantlets

Growth of orchids was evaluated by determining the length of shoot tips, number of shoots and roots/clump. First, the length (mm) of shoot tips were measured after cryopreservation and every 2 weeks for 8 weeks during reculturing. In addition, number of shoots and roots per clump of plantlets were determined from orchid plantlets after 12 weeks of reculturing.

Results

Effect of Dehydration on Water Content, Survival Ratio and Regrowth

The water content of all precultured beads continually decreased with increasing duration of air-dehydration. Survival ratio and regrowth values of encapsulated shoot tips also decreased with increased dehydration time as shown in Fig. 1.

Effect of Dehydration on Growth of Plantlets

Fig. 2 shows the length of shoot tips which were precultured on modified VW agar medium supplemented with 0.3 M sucrose for 2 d and then dehydrated in a sterile air flow for 0-10 h before reculturing. The length of shoot tips were slowly increased according to time of reculturing. However, they did not show any significantly different values ($P = 0.01$) among different dehydration times. In addition, the numbers of shoots and roots per clump were not affected by air-dehydration (data not shown).

Effect of Liquid Nitrogen on Survival Ratio and Regrowth

Fig. 3 shows the survival ratio and regrowth of encapsulated shoot tips which were precultured on 0.3, 0.5 and 0.7 M sucrose concentrations before dehydration and treated with and without LN. Both survival ratio and regrowth of all LN-treated beads were significantly lower than values of non LN-treated beads ($p = 0.01$). While the dehydration time increased, survival ratio of LN-treated from all preculture conditions were slowly increased. As the dehydration time (> 6 h) increased, the survival ratio values of encapsulated shoot tips were decreased until zero. In addition, increasing sucrose concentrations from 0.3 to 0.5 M in the precultured medium showed better survival compared with the control (0.029 M sucrose). Regrowth of LN-treated shoot tips were found after 8-10 h of dehydration when precultured on 0.3 M sucrose, and after 6 h of dehydration when precultured on 0.5 M sucrose. The highest survival ratio (16.18 mg living cells/100 mg total cells) and regrowth (13.33%) after cryopreservation were found when precultured on 0.3 M sucrose and after 6 and 8 h of dehydration, respectively.

Effect of Liquid Nitrogen on Growth of Plantlets

The length of encapsulated shoot tips after treatment

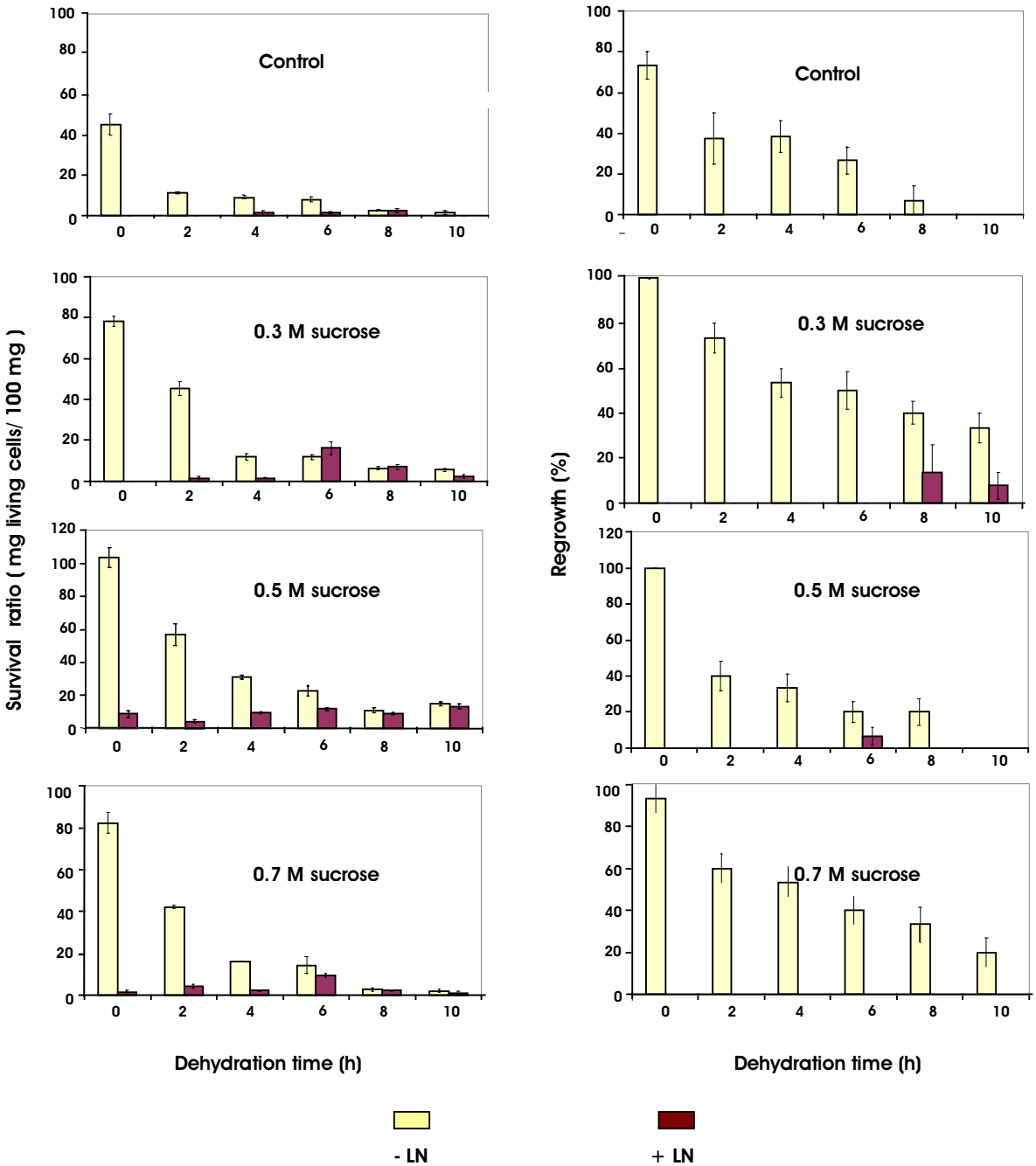

Fig 3. Effect of liquid nitrogen (LN) on survival ratio and regrowth of encapsulated shoot tips precultured on modified VW agar medium supplemented with 0.029 (control), 0.3, 0.5, and 0.7 M sucrose for 2 d and dehydrated for 0-10 h before treated with (+LN) and without liquid nitrogen (-LN). Vertical bars represent ± SD.

with and without LN were not significantly different ($p = 0.01$) as shown in Fig. 4. Also, numbers of shoots and roots per clump of encapsulated shoot tips after treated with and without LN counted from 12 weeks old of recultured plantlets were not significantly different (data not shown).

Morphological Characteristics after Cryopreservation

After cryopreservation, viable shoot tips turned

green within 7 d and increased in length by the 2nd-3rd week. The first pair of expanded leaves were observed by the 4th week and roots were developed after 6 weeks of reculturing. Some plantlets had more than one shoot per clump but all of them gave the green normal morphological characteristics (Fig. 5). After the plantlets were fully developed, they were transferred from flasks and grown in community pots, which were filled with charcoal (at the bottom) and osmunda fibres at the top, under 50% shade in the saranhouse (greenhouse)[2].

Discussion

Cryopreservation by encapsulation/dehydration was studied for shoot tips of *Dendrobium* Walter Oumae, which is a popular commercial cut-flower orchid of Thailand. This technique combined two cryoprotective treatments; namely precultured with sucrose and air dehydration. Both treatments have been used to investigate the optimal duration of dehydration, the most favorable sucrose concentrations and sucrose preculture for inducing cytoplasmic vitrification in order to avoid the formation of intracellular ice crystals during rapid cooling in LN.[14]

From the results, the survival ratio and regrowth of all encapsulated shoot tips decreased with decreasing bead water content and increasing dehydration time. There was a dramatic reduction in survival ratio and regrowth values between 0-2 h of dehydration, corresponding to rapid decrease of bead water content

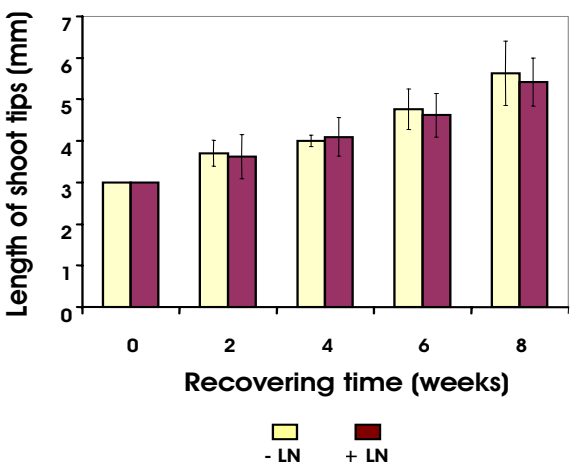

Fig 4. Effect of liquid nitrogen on length of shoot tips. Encapsulated shoot tips were precultured on modified VW agar medium supplemented with 0.3 M sucrose for 2 d and dehydrated for 8 h before treated with (+LN) and without liquid nitrogen (-LN). Vertical bars represent ± SD.

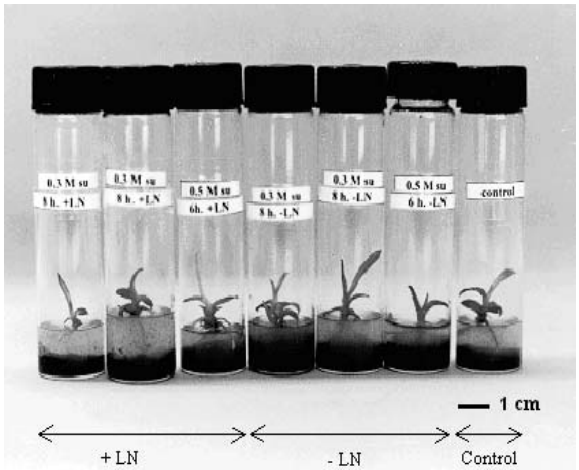

Fig 5. *Dendrobium* Walter Oumae plantlets recovered from cryopreservation at the 12th week.

(Fig 1). It suggests that when water was removed from the cell, it led to 'solute effect' such as pH changes, increasing electrolyte concentrations, protein denaturation, membrane phase transition and macromolecular interactions and then damage of the cell.[12, 15]

However, a dehydration step was necessary to avoid the formation of intracellular ice crystals even though increasing dehydration time led to damage of encapsulated shoot tips. Fig. 3 shows the effect of LN on the survival ratio and regrowth of orchid at various times of dehydration. Most of cryopreserved shoot tips (with LN treatment) gave significantly lower values than treated control (without LN treatment) ($p = 0.01$). It confirms that the damage of ice crystal occurs in the cells. However, increasing sucrose concentration in preculture treatment and dehydration times could improve the survival ratio and regrowth. Therefore, survival percentage of precultured beads after plunging into LN were slowly increased until 6-8 h of dehydration and then decreased again. Regrowth of encapsulated shoot tips after plunging into LN was observed after 6 h of dehydration from 0.5 M sucrose preculture condition, and after 8-10 h of dehydration from 0.3 M sucrose preculture condition. It indicated that 6-8 h of dehydration was sufficient to induce glass form of cryopreserved shoot tips.

The highest survival ratio (16.18±2.73 mg living cells/100 mg total cells) and regrowth (13.33±12.5 %) after cooling in LN were obtained from precultures with 0.3 M sucrose and after 6-8 h of dehydration (Fig 3). The low values may result from severe dehydration described earlier, and/or occur from uneven distribution of water in the tissue. Changrum *et al.*

(1999) suggested that the rate of water loss among different tissues. of various species, and even among tissues are variable.[15] Thus, drying may not be necessarily beneficial for cryopreservation, if uneven distribution of water results in different freezing responses among cells in the same tissue. Cells with high water content may be predisposed to the danger of intracellular ice formation and differential volumetric changes of cells would lead to considerable physical stress within the tissue.[6, 7, 15]

Precultured shoot tips on medium containing high sucrose concentration have been reported to show improved survival of cryopreserved shoot tips[8, 17, 18, 19] and this is confirmed by results in Fig. 3. The encapsulated shoot tips of *D*. Walter Oumae were precultured on 0.3, 0.5 and 0.7 M sucrose supplemented medium gave higher survival percentage after plunging into LN compared with the control (encapsulated shoot tips precultured on basal growth medium containing 0.029 M sucrose before cryopreservation). However, in some species such as coffee, hop and sugarcane, the use of very high sucrose concentration (1.0 M sucrose) was toxic for shoot tip survival.[20, 21, 22]

For cryopreservation of germplasm, it is particularly important that cryopreserved tissue can directly produce plants that are identical to non-treated phenotype.[15] In this experiment, the recovered orchid plantlets from cryopreservation by encapsulation/dehydration technique showed normal growth characteristics. The length of shoot tips after recovering from air-dehydration, LN-treatment increased slowly with times and at the same rate as the control plantlets (Fig. 4). Air-dehydration and LN did not affect the number of shoots and roots of regrowth plants. Orchid plantlets were successfully generated from cryopreservation by encapsulation/dehydration technique, and grew directly to be complete plantlets which comprise shoots, leaves and roots within 6 weeks after reculturing (Fig. 5). This suggests that many factors such as air-dehydration and LN did not affect growth and morphology of recovered shoot tips of *D*. Walter Oumae.

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