Plant Cell, Tissue and Organ Culture (PCTOC) (2018) 133:159–164
https://doi.org/10.1007/s11240-017-1363-8

RESEARCH NOTE

Cryopreservation of small leaf squares-bearing adventitious buds of *Lilium* Oriental hybrid 'Siberia' by vitrification

Chen Pan[1] · Jing Liu[1] · Wen-Lu Bi[1] · Haiyan Chen[1] · Florent Engelmann[2] · Qiao-Chun Wang[1]

Received: 8 June 2017 / Accepted: 26 November 2017 / Published online: 30 November 2017

Abstract

We report a new cryopreservation method for *Lilium* Oriental hybrid 'Siberia'. Adventitious buds were induced from leaf segments cultured for 12 days on adventitious bud induction medium composed of half-strength Murashige and Skoog medium (MS) supplemented with 1 mg L^{-1} α-naphthalene acetic acid and 0.5 mg L^{-1} thidiazuron. Small leaf squares (SLSs, 3 × 4 mm), each bearing at least one adventitious bud, were cut from leaf segments, precultured on medium with 0.5 M sucrose for 1 day, and then treated for 20 min with a loading solution containing 0.4 M sucrose and 2 M glycerol, followed by exposure to plant vitrification solution 2 for 7 h at 0 °C. Dehydrated SLSs were directly immersed in liquid nitrogen for 1 h. Cryopreserved SLSs were re-warmed in MS medium containing 1.2 M sucrose for 20 min at room temperature, followed by post-thaw culture for recovery. With this procedure, 85% survival and 72% shoot regrowth were achieved following cryopreservation. The use of SLSs bearing adventitious buds for cryopreservation reported in the present study eliminates the time-consuming and labour-intensive step of shoot tip excision, and has great potential to facilitate cryopreservation in other plant species.

Keywords Adventitious buds · Cryopreservation · Leaf segments · *Lilium* · Vitrification

Cryopreservation is considered an ideal means for long-term conservation of genetic resources. Although great progress has been made in plant cryopreservation over the last two decades, the continuous development of more efficient, wide-spectrum and cost-effective cryopreservation protocols would allow the much broader application of cryotechniques to the establishment of cryobanks. Efforts have been/are being exerted to address these issues. For example, droplet-vitrification, which derives from the droplet-freezing protocol described for *Manihot esculenta* shoot tips by Kartha et al. (1982), was further developed and applied to the entire *Musa* genetic diversity (Panis et al. 2005). With this protocol, a mean shoot regrowth rate of proximately 53% was obtained in 56 accessions belonging to eight different genomic groups of *Musa* spp. and one *Ensete* spp., containing edible diploid, triploid and tetraploid cultivars as well as wild diploids and synthetic hybrids. In the droplet-vitrification protocol, explants are treated with a loading solution, and then dehydrated by exposure to plant vitrification solution (PVS). After dehydration with PVS, the explants were placed in PVS droplets carried on a piece of aluminium foils, prior to direct immersion in liquid nitrogen (LN). Cryopreserved aluminium foils are then placed in liquid medium containing 1.2 M sucrose for re-warming and unloading, followed by post-thaw culture on recovery medium for regrowth. The main advantage of this technique is the possibility of achieving very high cooling/warming rates due to the very small volume of cryoprotective medium, in which the explants are placed, and to the direct contact between the explants and LN (cooling) and the unloading solution (rewarming) (Sakai and Engelmann 2007). The use of droplet-vitrification is capable of eliminating the genotype-specific response, which is a bottleneck to the establishment of plant cryobanks. Droplet-vitrification protocols have been developed for a large number of species including tuber crops such as *Solanum tuberosum* (Wang

Communicated by Sergio J. Ochatt.

✉ Qiao-Chun Wang
qiaochunwang@nwsuaf.edu.cn

[1] State Key Laboratory of Crop Stress Biology for Arid Areas, College of Horticulture, Northwest A&F University, Yangling 712100, Shaanxi, People's Republic of China

[2] IRD, UMR DIADE, 911 Avenue Agropolis, BP 64501, 34032 Montpellier cedex 5, France

et al. 2013) and *Colocasia esculenta* var. *esculenta* (Sant et al. 2008), fruit species such as *Malus* (Halmagyi et al. 2010; Li et al. 2015), vegetables like *Allium* (Keller et al. 2008) and ornamentals such as *Pelargonium* (Gallard et al. 2008) and *Lilium* (Yin et al. 2014).

The cryo-plate method, which combines the droplet-vitrification and encapsulation-vitrification procedures, was described for the first time by Yamamoto et al. (2011) for cryopreservation of *Tanacetum cinerariifolium* shoot tips. In this technique, explants are placed in the wells of an aluminium cryo-plate, to which they are made to adhere using small droplets of calcium alginate. In the V cryo-plate procedure, explants are treated with a loading solution, then with PVS before being directly immersed in LN (Yamamoto et al. 2011). In the D cryo-plate procedure, after treatment with a loading solution, explants are physically dehydrated in the air current of a laminar flow cabinet before direct immersion in LN (Niino et al. 2013). In addition to the high cooling and warming rates achieved due to the direct contact between the explants and LN during cooling, and with unloading solution during warming, a significant advantage of this technique lies with the easy handling of explants which adhere to the cryo-plates (Teixeira da Silva et al. 2015). To date, the cryo-plate procedure has been applied to almost 20 different species (Niino et al. 2017) including ornamentals such as *Dianthus caryophyllus* (Sekizawa et al. 2011), tuber crops such as *Solanum* spp. (Yamamoto et al. 2015), endangered species such as *Clinopodium odorum* (Engelmann-Sylvestre and Engelmann 2015) and fruit trees such as *Prunus* spp. (Vujović et al. 2015).

In all shoot tip cryopreservation protocols available now, including the droplet-vitrification and the cryo-plate (Panis et al. 2005; Yamamoto et al. 2011), excision of shoot tips is still a necessary step. This step requires skilled staff and is the most time-consuming and labour-intensive in the whole procedure (Harvengt et al. 2004). In addition, surgical excision of shoot tips from stock cultures may cause physical damage to and induce browning of explants.

We previously reported a droplet-vitrification protocol for cryopreservation of adventitious buds induced from leaf segments of *Lilium* (Yin et al. 2014). Here, we report the cryopreservation of small leaf squares (SLSs) bearing adventitious buds, which eliminates the need for excision of shoot tips, thus simplifying the cryogenic procedure and reducing labour costs.

The *Lilium* hybrid 'Siberia' was used in the present study. In vitro stock shoots were maintained on half-strength Murashige and Skoog (MS, 1962) medium supplemented with 1 mg L^{-1} 6-benzylaminopurine (BA), 0.2 mg L^{-1} naphthaleneacetic acid (NAA), 30 g L^{-1} sucrose and 7 g L^{-1} agar (pH 5.8), according to Yin et al. (2013). The cultures were grown in a growth chamber at 23 ± 2 °C under a 16 h light/8 h dark photoperiod with a light intensity of 45 µmol m^{-2} s^{-1} provided by cool-white fluorescent tubes. Subcultures were performed every 4 weeks.

Leaf segments, each 0.8–1.0 cm long and 0.4 cm wide, were excised from leaves of the third to sixth nodes of 4 week-old in vitro stock shoots and used for adventitious bud formation, according to Yin et al. (2013). Three transverse cuts were made across the leaf segments at1 mm intervals. Then, the leaf segments were cultured on adventitious bud induction medium (ABIM) composed of half-strength MS medium containing 1 mg L^{-1} NAA, 0.5 mg L^{-1} thidiazuron (TDZ), 30 g L^{-1} sucrose and 7 g L^{-1} agar (pH 5.8) in the light conditions described for in vitro stock shoots. Samples were taken from leaf segments after 9, 12 and 15 days of culture on ABIM, respectively, and used for histological observations, according to Yin et al. (2013). In brief, samples were fixed in formaldehyde acetic acid fixative (50% ethanol–formalin–acetic acid = 18:1:1) for 24 h, dehydrated through an incremental ethanol series (50, 70, 85 and 95%) and stored in 100% ethanol. After embedding in paraffin, 5-µm-thick sections were cut with a microtome (Leica RM2235, Leica Microsystems, Heidelberg, Germany) and stained with 0.01% toluidine blue. The sections were observed with a light microscope (Leica DM2000, Leica Microsystems, Heidelberg, Germany). Samples harvested at the same time points of adventitious bud induction were used for cryopreservation, as described by Yin et al. (2014). SLSs, each being 3 × 4 mm in size and containing at least one adventitious bud (Fig. 1a) were cut from the leaf segments and precultured on solid MS containing 0.5 M sucrose for 1 day in the same conditions, as used for the in vitro stock shoots. Precultured shoot tips were osmoprotected in a loading solution containing 2.0 M glycerol and 0.4 M sucrose for 20 min at room temperature. Then, loaded shoot tips were exposed to PVS2 (Sakai et al. 1990) at 0 °C for time durations ranging from 4 to 9 h. PVS2 contains MS medium supplemented with 30% (w/v) glycerol, 15% (w/v) ethylene glycol, 15% (w/v) dimethylsulfoxide and 0.4 M sucrose (Sakai et al. 1990). At the end of PVS2 dehydration, SLSs were directly dropped into cryovials (five SLSs per 1.8 ml cryotube) that had been previously filled with LN and stored in LN for 1 h. Rewarming was performed by taking the cryovials out of LN, and then SLSs were immediately removed from the cryovials and placed in an unloading solution composed of MS containing 1.2 M sucrose and incubated at room temperature for 20 min. Unloaded SLSs were then post-thaw cultured in a Petri dish (90 mm in diameter) containing 25 ml MS medium supplemented with 1 mg L^{-1} NAA and 0.2 mg L^{-1} TDZ. The cultures were grown at 23 ± 2 °C in the dark for 3 days and then transferred to light conditions for survival and shoot regrowth. Samples that received all treatments except LN exposure served as the treated control (−LN). Shoot tips exhibiting green colour after 7 days of post-thaw culture were considered surviving,

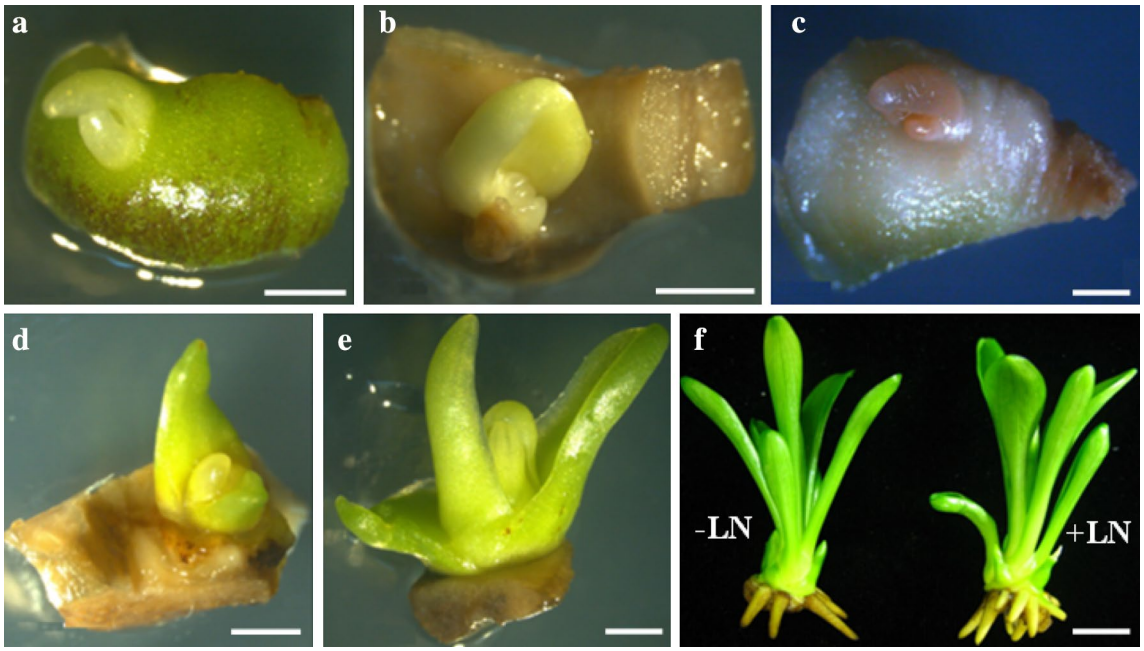

Fig. 1 Survival and shoot regrowth of cryopreserved adventitious buds produced on small leaf squares (SLSs) of *Lilium* Oriental hybrid 'Siberia'. A SLS bearing one adventitious bud used for cryopreservation (**a**). Bud that withstood LN exposure after 7 days of post-thaw culture (**b**). Dead bud following cryopreservation (**c**). Shoot elongation from cryopreserved bud after 14 days of post-thaw culture (**d**). Shoot regenerated from cryopreservation after 28 days of post-thaw culture (**e**). Plantlet regenerated from the control (−LN) and from cryopreserved adventitious buds (+LN) after 28 days of culture on rooting medium (**f**). Bars in **a**, **b**, **c**, **d** and **e** = 1 mm, and in **f** = 1 cm

while shoot regrowth was defined as surviving shoot tips that regenerated into shoots (≥ 5 mm in length) after 4 weeks of post-thaw culture. For rooting, shoots (≥ 5 mm in length) were transferred on rooting medium composed of MS supplemented with 1 mg L^{-1} NAA and cultured in the same conditions as used for in vitro stock shoots.

At least 10 samples from each treatment of two independent experiments were used for histological studies. For cryopreservation experiments, 10 shoot tips were used in each of three replicates. All experiments were repeated twice. Data expressed as percentage with their standard error were transformed using arc sine prior to analysis of variance (Compton 1994) and Student's *t*-test. Least significant differences were calculated at $P < 0.05$.

Small protuberances (Fig. 2a), each having an apical dome and at least two leaf primordia (LPs) (Fig. 2b), were visible on leaf segments after 9 days of post-thaw culture. These protuberances continued growing into adventitious buds (Fig. 2c) with about 4–5 LPs (Fig. 2d) after 12 days of post-thaw culture, and started elongating into shoots (Fig. 2e, f) after 15 days of post-thaw culture. For the treated control (−LN), all SLSs survived, regardless of the age of adventitious buds (Fig. 3a). Shoot regrowth rate was lower in 9-day-old SLSs than in 12- and 15-day-old ones (Fig. 3b). Following cryopreservation (+LN), survival and regrowth rates were significantly lower in 9-day-old SLSs than in 12- and 15-day-old ones (Fig. 3a, b). Although there was no significant difference in survival between 12- (85%) and 15-day-old SLSs (83%), shoot regrowth rate was significantly higher in the former (72%) than in the latter (54%) (Fig. 3b). The duration of exposure to PVS2 affected recovery of the treated control (−LN) and cryopreserved samples (+LN). For the treated control (−LN), survival rates did not vary significantly, remaining between 95 and 100% during 4–9 h of exposure to PVS2 (Fig. 4a). Shoot regrowth rates decreased progressively from 100% (4 h) to about 80% (9 h) (Fig. 4a). Following cryopreservation (+LN), similar patterns were found in survival and shoot regrowth rates (Fig. 4a, b). Survival rates increased from about 50% (4 h) to 84% (7 h), and then decreased to about 70% (9 h) (Fig. 4a). Shoot regrowth rates increased from 44 to 72% as exposure time to PVS2 increased from 4 to 7 h, and then decreased to 42% (9 h) (Fig. 4b). Surviving cryopreserved adventitious buds showed green color after 7 days of post-thaw culture (Fig. 1b), while dead ones became brown (Fig. 1c). Surviving buds started to elongate and developed into shoots (≥ 5 mm) after 14 (Fig. 1d) and 28 days (Fig. 1e) of post-thaw culture, respectively. Whole plantlets with roots were produced after 21 days of culture on rooting medium (Fig. 1f). The morphology of plantlets recovered from cryopreservation was identical to that of the untreated controls (Fig. 1f).

In the present study, we demonstrated that Lilium hybrid 'Siberia' SLSs bearing adventitious buds could be

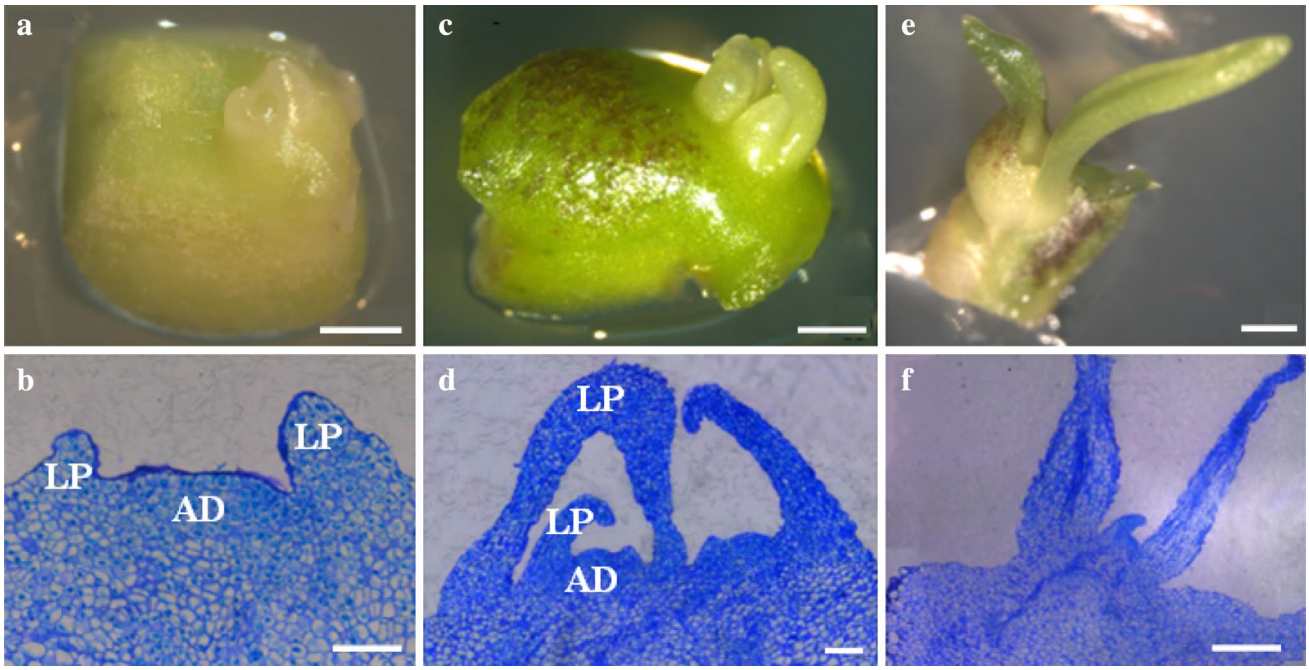

Fig. 2 Morphological and histological observations of adventitious bud formation on SLSs of *Lilium* Oriental hybrid 'Siberia'. Leaf segments were grown on half-strength MS medium containing 1 mg L^{-1} NAA and 0.5 mg L^{-1} thidiazuron (TDZ), and placed at a consistent temperature of 23 ± 2 °C under a 16 h light/8 h dark photoperiod. Nine- (**a**, **b**), 12- (**c**, **d**) and 15- (**e**, **f**) day-old adventitious buds. *AD* apical dome, *LP* leaf primordium. Bars in **a**, **c** and **e** = 1 mm, in **b** and **d** = 20 µm, and in **f** = 0.5 mm

cryopreserved by vitrification protocol, resulting in 85% survival and 72% shoot regrowth. We found that the age of adventitious buds affected recovery of cryopreserved samples and that the highest recovery was achieved with 12-day-old buds. Li et al. (2014) reported that recovery of cryopreserved *Malus* adventitious buds increased dramatically as their age increased from 9 to 11 weeks, and then decreased as bud age further increased (Li et al. 2014). The age of stock cultures was found to affect recovery of cryopreserved shoot tips in other plant species such as *Solanum tuberosum* (Halmagyi et al. 2005) and *Chrysanthemum morifolium* (Wang et al. 2014). The suitable age of stock cultures for obtaining optimal recovery may vary with plant species and explant type. In our previous study using the same lily hybrid and the same protocol for adventitious bud regeneration, the optimal duration of exposure of shoot tips to PVS2 was 4 h (Yin et al. 2014), which was much shorter than 7 h reported here. The exposure time to PVS varies with the size and structure of cryopreserved explants and appears to be highly species-specific (Sakai and Engelmann 2007). The SLSs (3 × 4 mm) used in the present study were much larger than shoot tips (1.5–2 mm in length) used in the study of Yin et al. (2014), thus requiring longer exposure time to PVS2.

In vitro adventitious shoot regeneration has been widely used for micropropagation and genetic transformation in a large number of plants including *Lilium* (Nhut et al. 2001; Motte et al. 2014; Dobránszki and Teixeira da Silva 2010; Yin et al. 2013). Recently, leaf segments-derived adventitious buds have also been successfully cryopreserved in several plants such as *Begonia* × *erythrophylla* (Burritt 2008), *Lilium* (Yin et al. 2014) and *Malus* (Li et al. 2014). The major concern when using adventitious regeneration is the genetic stability in in vitro regenerants. With proper manipulations such as application of growth regulators and choice of explants type, genetic stability can be maintained in the regenerants (Nhut et al. 2001; Motte et al. 2014; Dobránszki and Teixeira da Silva 2010; Yin et al. 2013; Li et al. 2014). In *Lilium* Oriental hybrid 'Siberia' we previously reported that only 0.73% polymorphic bands were detected by amplified fragment length polymorphism (AFLP) in in vitro regenerants (Yin et al. 2013), while no polymorphic bands were detected by inter-simple sequence repeat markers (ISSR) in the regenerants recovered from cryopreserved shoot tips. Moreover the morphology of regenerants recovered from cryopreserved shoot tips was identical to that of the control in the study of Yin et al. (2014) and in the present study.

Compared with traditional methods, the use of in vitro-derived adventitious buds significantly improved the efficiency of shoot tip production for cryopreservation (Yin et al. 2013; Li et al. 2014). Cryopreservation of SLSs bearing adventitious buds reported in the present study added extra value to plant cryopreservation by eliminating excision of shoot tips, which requires skillfull staff, and is the most

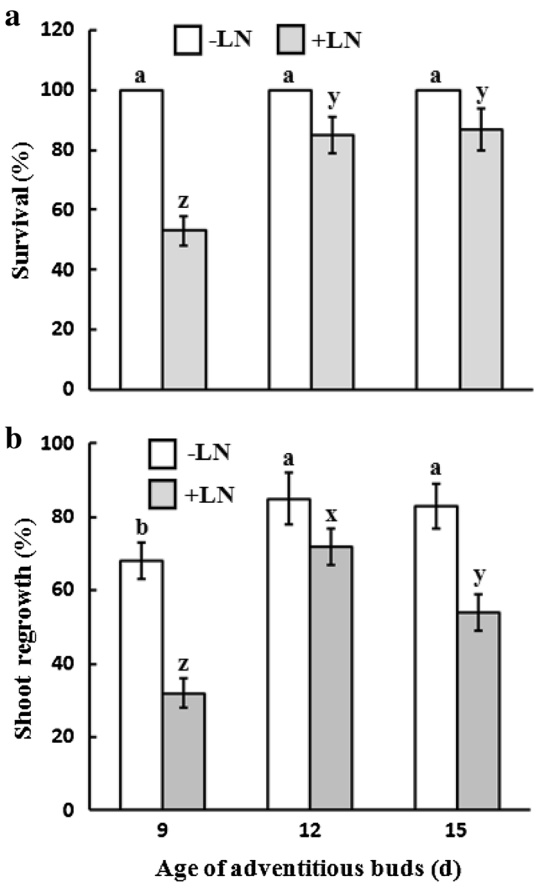

Fig. 3 Effects of age on survival (**a**) and shoot regrowth (**b**) from the control (−LN) and cryopreserved (+LN) small leaf squares (SLSs) *Lilium* Oriental hybrid 'Siberia'. Time of exposure to PVS2 was 7 h at 0 °C. Data are presented as means ± SE, and with different letters in the same parameter indicate significant differences at $P < 0.05$ analyzed by Student's *t*-test

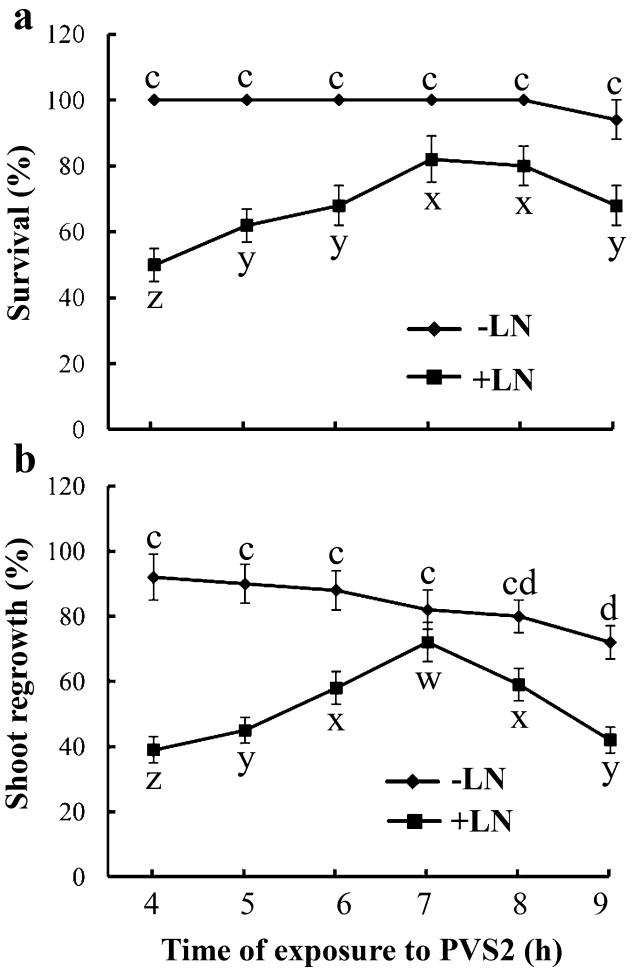

Fig. 4 Effects of time of exposure to PVS2 on survival (**a**) and shoot regrowth (**b**) from the treated control (−LN) and cryopreserved (+LN) small leaf squares (SLSs) of *Lilium* Oriental hybrid 'Siberia'. Twelve-day-old buds produced on SLSs were used. Data are presented as means ± SE and with different letters in the same parameter indicate significant differences at $P < 0.05$ analyzed by Student's *t*-test

time-consuming and labour-intensive step of cryogenic procedures (Harvengt et al. 2004). Therefore, this new protocol has potential to facilitate cryopreservation of plant species, in which protocol is available of high efficient shoot regeneration from leaf segments and genetic stability in the regenerants can be maintained.

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