Pak. J. Bot., 46(1): 307-315, 2014.

THE EFFECTS OF SUCROSE CONCENTRATION AND LIGHT CONDITION ON LILY'S BULBLET-IN-TUBE PRODUCTION AND INCLUSION CONTENT

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Abstract

The effects of sucrose concentration and light condition on bulblet-in-tube's regeneration were investigated. Results revealed that explants ('Siberia' bulb scale) cultured on media containing 3 $g.L^{-1}$ sucrose induced fast (23.33 days) with the highest inducement rate (88.91%) and most buds (13.78/explants). In 75 days of swelling period, the diameter of bulblet on media with 8 $g.L^{-1}$ sucrose increased most (350.44%) with best roots formation. Explants provided with 16-h photoperiod light condition (L) differentiated fast (27.60 days) with highest proliferation multiple (6.4). Besides, most of them (67.36%) induced adventitious buds. While plants in continuous darkness condition (D), most of explants (78.69%) developed bulblets directly. After swelling, bulblets induced and swelled in D had the highest diameter increase rate (443.70%) with most (14.53 roots/bulblet) and longest roots (36.82 mm). Interestingly, the bulblets differentiated in L and swelled in D (L-D) only increased to 245.33% with least (7.31 roots/bulblet) and shortest roots (7.96 mm). Carbohydrate contents of bulblet cultured in various light conditions showed significant difference. L condition applied in all phases was best for carbohydrate accumulation of bulblets and L-D (differentiate in light and swell in dark condition) was worst. Endogenous hormones contents of bulblets provided with different light conditions showed slight difference. During dormancy breaking period, all index above exhibited similar change trend with lily commercial bulb and there was slight difference among different bulblets. *Ex vitro* performances of those bulblets were also compared. The surviving rate in L-L (differentiate in light and swell in light condition) was 66.7% which was also the highest immediately followed by bulblet in D-D (Differentiate in dark and swell in dark condition) (53.3%). The bulblets in L-D survived least (26.7%).

Introduction

Lily, due to its large, colorful and attractive flowers, has been one of the most important commercial cut-flower species and one of the three major bulb crops in the commercial market (Robinson & Firoozabady, 1993). The common method to achieve new cultivars with good characters is through conventional breeding which is hampered by its heterozygous state and self-incompatibility among the species of the different *Lilium* groups (Van Tuyl *et al*., 1992). Thus, *In vitro* embryo rescuing protocol was developed to achieve rapid mass production and fast regeneration of uniform plant material, and therefore has been recognized as a necessity particularly for future breeding and culture of lilies (Pelkonen, 2005). Besides, for conservation and commercial exploitation of the species, an efficient *In vitro* production protocol is desirable (Priyadarshi & Sen, 1992; Wickremesinhe *et al.*, 1994; Wawrosch *et al*., 2001; Muhanmmad *et al.*, 2013). In recent years, many non-conventional breeding approaches and further studies have been developed in order to offer new promising strategies for the improvement of lily as well as other plants (Khalid *et al.,* 2013.). Most of these studies also require a reproducible *In vitro* regeneration and propagation protocol as basis.

Plant regeneration of lily via organogenesis was achieved from a vast array of explants ranging from flower organs to bulb scales. Apart from explants type, the constitution of different basic media (Mudassir *et al.*, 2012.**)** various plant growth regulators like NAA, BA (Muhammand & Faheem 2012.), 2, 4-D (Imran *et al.,* 2012) and other supplement substance applied in other species like Calcium chloride (Noorul *et al.*, 2013.), BAP (Reza *et al.,* 2012.) **i**n different concentrations were the main factors that affect tissue culture efficiency of lily. Efficient bulblet-in-tube production is essential for lily study. However, the effects of sucrose concentration and, especially light condition on bulblet-in-tube's regeneration have been controversial. The effect of sucrose concentration on shoot or plant regeneration and bulb swelling had been shown in other lily species (Takayama *et al*., 1980; Langens-Gerrits *et al*., 1992; Bonnier & Van Tuyl, 1997). There were some reports suggested that medium with high concentration of sucrose were suitable for bulblet growth (Bonnier & Van Tuyl, 1997; Langens-Gerrits *et al.,* 1997; Marinengeli & Curvetto, 1997; de Klerk *et al.,* 1992; Witomska *et al.*, 2000; Joshi & Dhar, 2009; Bakhshaie *et al.,* 2010). As far as light condition was concerned, normally, the plantlets were cultured under 2000 lux light at a 16-photoperiod. But there are studies on influence of darkness condition on lily micro propagation (Zhao *et al.*, 2010; Mei-Lan, L. *et al.,* 2003). *In vitro* regenerated bulblets are reported to exhibit dormancy (Harvey & Selby, 1997; Langens-Gerrits *et al.,* 2001), which was controlled by the factors such as temperature, sucrose and ABA (Aguettaz *et al.,* 1990; Delvallée *et al.,* 1990; Djilianov *et al.,* 1994; Kim *et al.,* 1994).

Based on all above studies, we made following assumption: Does sucrose plays similar effect during both differentiation and swelling on bulblet culture? Is the light an indispensable factor during each phase? Would the *ex vitro* performance of the bulblets obtained under different light conditions be the same? During mass production of tissue culture seedling nowadays, the fund for plant growth regulator and especial energy consuming for light was a large part of production cost and not quite suitable for energy conservation. Therefore, the objective of this present study was to figure out whether we can just manipulate sucrose concentration to affect explants differentiation efficiency. Furthermore, during different culture phases, whether light condition is essential for *In vitro* culture. During dormancy breaking period, whether there is significant difference among the bulblet produced under different light conditions. Accordingly, this research would not only establish a highly efficient bulb-in-tube culture system which was the basis of all further research on lily, but also focus on getting a better understanding of the role sucrose and light plays in explants' differentiation and bulblet morphogenetic formation.

Materials and Methods

Plant material and culture condition: Donor materials were Lily bulb 'Siberia' (cv. Oriental) imported from Holland. The intermediate scales of the commercial bulbs were used as the first explants. The pH of all the media was adjusted to 6.0 with 1.0 N HCl or 1.0 N NaOH before adding 0.7 % agar. The media was then dispensed into conical flasks after autoclaving for 20 min at 121℃, 138 kPa. The explants were cultured in a growth chamber at 25±1℃ and 70–80% relative humidity (RH) with cool white light provided by Topstar cool whiteTSZJD2/T5 fluorescent lamps (28 W).

Shoots and bulblets induced on media with different sucrose concentrations: The bulb scales were excised and washed in tap water to remove substrate before taken into clean bench where they were surface-sterilized in 70% ethanol (30s), rinsed once in sterilized water for 3 min, then shaken for 10 min in 10% (v/v) sodium hypochlorite solution and rinsed three times in sterilized water. The next step was to soak up the extra water of bulb scales and cut them into segments about 5 × 5 mm consisting of the basal part placed on Murashige and Skoog (MS) basal medium (Murashige & Skoog, 1962).

After 7 days cultured in MS basal medium with no plant growth regulators (PGRs), the explants were then placed onto MS media containing 1.07 μM NAA (α-Naphthaleneacetic acid) and 4.44 μM 6BA (6-Benzyladenine) separately supplemented with 30, 60 and 90 $g.L^{-1}$ sucrose. Three replication were prepared for each treatment with n=15. Average time of inducement, rate of induction and proliferation multiple were investigated and analyzed to find out the effects on differentiation of various sucrose concentrations.

Effects of different light conditions on differentiation phase: In order to find out the effects of light on explants' differentiation, all cultures were separately incubated in a 16- photoperiod (L) and continuous darkness (D). Apart from that, all other culture conditions remained the same. The medium format was MS media containing 1.07 μM NAA and 4.44 μM supplemented with 30 $g.L^{-1}$ sucrose. Average time of inducement, rate of induction and proliferation multiple were also investigated separately.

Effects of different sucrose concentrations on bulblets swelling: After 3 weeks in culture, similar-sized shoots (2-3 mm diameter) that differentiated from the *In vitro* bulb scale segments were separated and cultured on MS medium supplemented with 60, 70, 80 and 90 $g.L^{-1}$ sucrose with no PGRs in light condition. The proliferation multiples, increasing rate of bulblet's diameter and number of roots per explants were investigated. This experiment were repeated three times (n=15).

Bulblets swelling under different light conditions: In this part of experiment, three combination which were shoots differentiated in ordinary light condition (16-h photoperiod) and swelled under the same light condition (L-L) or transfer to continuous darkness (L-D) and shoots differentiated in continuous darkness swelled in darkness too (D-D) were carried out separately. The media were MS only supplemented with 80 $g.L^{-1}$ sucrose. This part of experiment lasted for 75 days.

Effects of light conditions on bulblets carbohydrate content during cold storage: In this experiment, carbohydrate content of bulblets cultured in different light conditions with similar size was measured. And then all bulblets were stored in 4℃ to break the dormancy. Content of soluble sugar, reducible sugar and starch determination through DNS test (Miller, 1989) were carried on every week for 4 times in total during this period with three samples for each treatment.

Effects of light conditions on bulblets endogenous hormone content during cold storage: The exaction and purification of plant growth regulator referred to the method of Lin (1975). We changed the filtration during exaction process into low temperature centrifuge to improve exaction efficiency. This part was also carried on every week for 4 times in total during cold storage with three samples for each treatment.

Agilent 1100 series HPLC was used to detect the endogenous hormone changes during cold storage of tissue cultured bulblet. The standard sample of IAA (Indole-3-acetic acid), GA_{1+3} (Gibberellin A 1 and 3) and ABA (Abscisic Acid) were from Sigma Company. Chromatographic conditions were as follows: Agilent 1100 series HPLC, Agilent C18 column (250 × 4.6 mm × 5 μm). Mobile phase of IAA, GA_{1+3}, ABA detection was mixture of ACS-0.1 M acetic acid (3:97, V: V). Column unit temperature was 30℃ with diode array detector. Detection wavelengths were IAA-280 nm, ABA-260 nm, and GA_{1+3}-210 nm. The flow rate was $1ml.min^{-1}$. This experiment was repeated three times for each treatment.

Transplantation of bulblet cultured through micro propagation: After 4 weeks storage at 4℃, 45 bulblets swelled under different light conditions with well-developed roots were transplanted into a greenhouse in 5 cm plastic pots containing a mixture of peat and perlite in ratio of 2:1. The daily managements which were most suitable for lily tissue culture bulblet were applied. This part of experiment took place in May.

Statistical analysis: Data was recorded and statics were analyzed through SPSS v. 18.0. Means were analyzed by Analysis of Variance. Significant differences between means were compared by the Least Significant Difference (LSD) test and the means for the different traits were subsequently separated by a Duncan test using. For all comparisons, statistical significance was considered at P= 0.05 and 0.01.

Results

Effects of different sucrose concentration on differentiation: The different responses of explants cultured in media with different concentrations of sucrose were observed during the differentiation period. Three indexes which were the average time of inducement, induction rate and proliferation multiple showed very significant differences. According to the statistics (Table 1), the highest induction rate (88.91%) and proliferation multiple (13.78) occurred in 30 $g.L^{-1}$ sucrose media supplemented with 1.07 μM NAA and 4.44 μM 6-BA which had the shortest inducement time (23.33 days on average). With the rise of sucrose concentration, the time for bulb scale to differentiate was prolonged. Meantime, the rate of induction and proliferation multiple descended. As for 60 $g.L^{-1}$ sucrose, it took average 35.78 days to induce, the average induction rate was 75.00% with 8.29 proliferation multiple. The medium with 9% sucrose had the lowest induction rate (63.00%) and proliferation multiple (3.72) with the longest inducement time (42.76 days on average).

Explant cultured under different light conditions in differentiation phase: Light condition had a significant impact on differentiation. When it was continuous darkness, longer induction time (32.40 days) was needed (Table 2). The rate of induction (81.30%) was considerable and exhibited a lower proliferation multiple (5.3) While, when explants cultured under a 16-h photoperiod had shorter induction time (27.60 days), slightly higher rate of induction (84.90%) and proliferation multiple which is 6.40. Moreover, various differentiation types were noticed during this phase. And the difference of differentiation type under different light condition was signifant. Most bulb scales segments turned green after 10 days cultured in 16-h photoperiod. From statistics in Table 2, it was observed that mainly adventitious buds (67.36%) with many leaflets that were induced (Fig. 1a). While explants remained white in darkness condition and lots of well-formed bulblets (78.69%) with clasping bulb scales were induced directly from explants (Fig. 1b).

Impacts of various sucrose concentrations on bulblets swelling: In this experiment, about the same size bulblets were cultured in MS media only with 60, 70, 80, 90 $g.L^{-1}$ sucrose. The bulblet swelling showed significant difference among various media after 75 days (Table 3). Along with the increment of sucrose concentration, the proliferation multiple decreased distinctly from 5.11 to 0.64. For 80 $g.L^{-1}$ sucrose in media, the proliferation multiple was 3.70 which showed no significant difference with media containing 70 $g.L^{-1}$ (3.11). The largest increasing rate of bulblet's diameter was 350.44% which occurred in media supplemented with 80 $g.L^{-1}$ sucrose while the remaining concentrations showed no significant difference on this index which ranged from 260.00 - 267.33%. Media with 60 $g.L^{-1}$ sucrose had 10.28 roots grown per bulblet. And on media with 80 $g.L^{-1}$ sucrose, every bulblet had 8.77 roots on average. There was no significant difference between them through Duncan test. Following up was average 6.23 roots grew from every bulblet on media containing the 90 $g.L^{-1}$ sucrose. The fewest roots occurred on 70 $g.L^{-1}$ sucrose which was 4.11 per bulblet.

Table 1. Effects of various sucrose concentrations on differentiation of bulb scale.

Concentrations of sucrose ($g.L^{-1}$)	Average time of inducement (days)	Rate of induction (%)	Proliferation multiple
30	23.33cC	88.91aA	13.78aA
60	35.76bB	75.00bB	8.29bB
90	42.76aA	63.00cC	3.72cC

Rate of induction = Number of induced explants / Total explants × 100%, Proliferation multiple = Number of shoots / Total explants, time of inducement included the time of transition culture in MS media (7 days) and time to induce shoots
Means followed by different letters within columns are significantly different at P= 0.05 (lowercase letter) and 0.01 (capital letter) through Duncan test
ht conditions in differentiation phase

Table 2. Effects of different light conditions on differentiation of bulb scale.

Light conditions	Average time of inducement (days)	Rate of induction (%)	Proliferation multiple	Rate of shoots induction (%)	Rate of bulblet induction (%)
D	32.40	81.30	5.30	21.31	78.69
L	27.60	84.90	6.40	67.36	32.64
F	17.411*	14.897*	181.500**	2481.591**	3438.071**
Sig.	0.014	0.018	0.000	0.000	0.000

D: continuous darkness; L: 16-h photoperiod. Rate of shoots / Bulblet induction = Number of explants induce shoots or bulblet/ Number of total explants
F value indicates significant difference within the column following ANOVA (* was significant different when p=0.05, ** was when p=0.01)

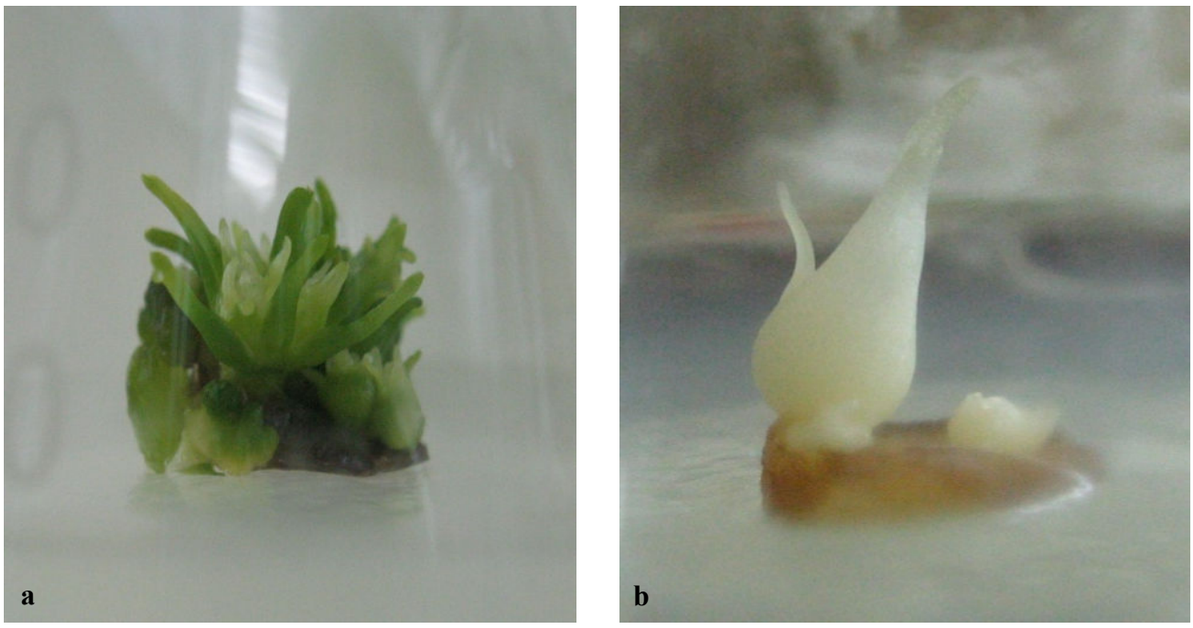

Fig. 1 Induction of bulb scales under different light conditions
(a): bulb scale cultured under 16h photoperiod; (b): bulb scale cultured under continuous darkness

Influence of light conditions on bulblets-swelling: The bulblets in similar size were removed to different light conditions in MS supplement with 80 $g.L^{-1}$ sucrose. The results were revealed in Table 4. Accordingly, bulblet swelling appeared to be different under different light conditions (Fig. 2a-c). The shoots cultured in continuous darkness grew into the largest bulblet diameter increase rate (443.70%) with longest roots (36.82 cm) and induced most roots (14.53 per bulblet). Following up were bulblets swelling under 16-h photoperiod. They had second large diameter increase rate (384.44% on average), roots number (7.80) and roots length (27.87 cm). Most interestingly, the bulblets differentiated in darkness and swelled in light were the smallest diameter increase rate (245.33%) with less (7.31 roots/bulblets) and shorter roots (7.96 mm on average).

The effects of light conditions on bulblets carbohydrate content during cold storage: Initially, the well swelled bulbs with well-developed roots were transferred directly after hardening off to a greenhouse. It appeared that those bulbs were at their dormancy stage. Subsequently, we stored them in 4°C aiming at breaking the dormancy. During this period, we monitored their carbohydrate content. From data in Table 5, the carbohydrate content of bulblet cultured under various light conditions showed significant difference before cold treatment. The bulblet cultured through L-L condition synthetize more carbohydrate including soluble sugar (41.77 mg.g FW^{-1}), reducing sugar (89.30 mg.g FW^{-1}) and starch (279.23 $mg.gFW^{-1}$) content. Following up was the bulblet in L-D condition. Under D-D condition, the carbohydrate content was the lowest. This character maintained the same during 4 weeks of cold storage. It was known that plants turned starch into soluble sugar which can be directly used during cold storage in order to break dormancy and get ready for next season growth. The results of this experiment showed that the starch contents in tube-cultured bulblets decreased (Fig. 3c) and soluble sugar contents increased during 4°C cold storage for 4 weeks (Fig. 3a) which indicated that the dormancy was on the way of breaking during the cold storage. From Fig. 3, it revealed that the carbohydrate change trends of all three different light conditions were in same pattern.

The effects of culture conditions on bulblets endogenous hormone during cold storage: The changes of three endogenous hormones were also monitored during cold storage. From Table 6, the IAA content of bulblets that cultured in three light conditions showed significant difference. Under L-L condition, bulblet had highest IAA content (76.33 $ng.gFW^{-1}$) followed by D-D condition (74.50 ng.g FW^{-1}). The lowest IAA content was bulblet cultured in L-D condition (72.83 ng.g FW^{-1}). As for GA_{1+3} and ABA content, the bulblet produced under different light condition showed slight difference. Along with the exceeding of cold storage, the endogenous hormones content tended to narrow their difference among bulblets cultured in L-L, L-D and D-D conditions. The results also showed that IAA content were declining in first week then rising up (Fig. 4a). Apart from bulblet cultured under L-D condition, GA1+3 content also increased (Fig. 4b) while ABA content decreased (Fig. 4c). According to Li Yihua's study (2011), the value of GA_{1+3} /ABA content was an important index to determine the breaking of bulb dormancy. As in commercial bulb, the value usually reached its highest which was nearly 4 after 8 week of cold treatment (Gude *et al.,* 2000; Roh, 1982; Zhang, 2010). It was noticed that after 4 weeks cold storage, the GA_{1+3} /ABA value of bulblet cultured in different light showed no significant difference (Table 6). We could assume that the dormancy breaking process of bulblet produced under various light conditions were quite similar.

Table 3. Impacts of various sucrose concentrations on bulblet swelling.

Concentrations of sucrose ($g.L^{-1}$)	Proliferation multiple	Average bulblet's diameter increasing rate (%)	Average roots per bulblet
60	5.11aA	260.00bB	10.28aA
70	3.70bB	266.78bB	4.11bB
80	3.11bB	350.44aA	8.77aA
90	0.64cC	267.33bB	6.23cC

Proliferation multiple = Shoots / Total bulblet × 100%, average bulblet's diameter increasing rate = Final diameter / Previous diameter × 100%, average roots per bulblet = Root number per bulblet

Means followed by different letters within columns are significantly different at p = 0.05 (lowercase letter) and 0.01 (capital letter) through Duncan test

Table 4. Effects of various light conditions on bulblet swelling.

Light condition	Increasing rate of bulblet's diameter (%)	Average roots per bulblet	Average length of roots per bulblet (cm)
L-L	384.44bB	7.80bB	27.87bB
L-D	245.33cC	7.31bB	7.96cB
D-D	443.70aA	14.53aA	36.82aA

L-L: Shoots which differentiated under in 16-h photoperiod was swelling under the same light condition; L-D: Shoots which differentiated under ordinary light (16-h photoperiod) was swelling under darkness; D-D: Shoots which differentiated under continuous darkness was swelling under darkness too

Average roots per bulblet = Number of roots in total / Number of bulblet in total, Average length of roots per bulblet = Length of all roots / Number of bulblet in total

Means followed by different letters within columns are significantly different at p= 0.05 (lowercase letter) and 0.01 (capital letter) through Duncan test

Table 5. Carbohydrate contents of bulblets cultured under different light conditions.

Light conditions	Soluble sugar (mg. g FW^{-1})	Reducing sugar (mg. g FW^{-1})	Starch (mg. g FW^{-1})
L-L	41.77aA	89.30aA	279.23aA
L-D	36.67bB	73.77bB	257.43bB
D-D	29.73cC	58.77cC	221.47cC

Means followed by different letters within columns are significantly different at p= 0.05 (lowercase letter) and 0.01 (capital letter) through Duncan test

Table 6. Different endogenous hormone contents of bulblet cultured under different light conditions.

Light conditions	IAA (ng. g FW^{-1})	GA_{1+3} (ng. g FW^{-1})	ABA (ng. g FW^{-1})	GA_{1+3}/ABA
L-L	76.33aA	91.33abA	109.67bB	0.77aA
L-D	72.83cC	90.20bA	111.97aA	0.77aA
D-D	74.50bB	92.07aA	113.20aA	0.83aA

Means followed by different letters within columns are significantly different at p= 0.05 (lowercase letter) and 0.01 (capital letter) through Duncan test.

Acclimatization of bulblets under different light conditions: After a period of 4 weeks at 4°C, 45 in total of the bulblets cultured in different conditions with well-developed roots were transplanted into a greenhouse (15 bulblets per condition). 10 of the bulblets induced and swelling under light (L-L) survived and germinated which was the highest surviving rate after transplantation (66.7%). Following closely up was those under D-D condition that was 8 sprouted out of 15 (53.3%). Most of the bulblets produced under L-D condition corrupted and only 4 germinated (26.7%).

Discussion

Effects of sucrose concentration: The regulation of sucrose concentrations is regulating carbon-nitrogen ratio, the carbohydrate accumulation capacity affects bulblet growth strongly. Bulb growth *In vitro* depends on the sucrose concentration in the media (Langens-Gerrits *et al.,* 1997; Langens-Gerrits *et al.*, 2003). Sucrose at 4.5% (w/v) concentration exhibited maximum regeneration and produced higher number of bulblets per explant on both callus and bulblet scale explants than the other concentrations tested for *Lilium oxypetalum* (Joshi & Dhar, 2009).

In the present research, concentration of sucrose had different impact on explants, especially during different development phase. As for differentiation of lily *In vitro* condition, lower concentration of sucrose in media was more suitable for higher shoot inducement rate and shorter time. 30 $g.L^{-1}$ of sucrose was believed to be the best concentration as 88.91% on average of explants differentiated at 23.33 days and had 13.78 shoots on average grown from each bulb scales (Table 1). Along with the increment of sucrose in media, the shoot inducement rate and proliferation decreased which was consistent with some previous study (Bonnier & Van Tuyl, 1997; Nhut *et al.*, 2001). Besides, several deformities of shoots were found on media containing 90 $g.L^{-1}$ of sucrose. This would prove that high sucrose concentration suppress adventitious shoots inducement.

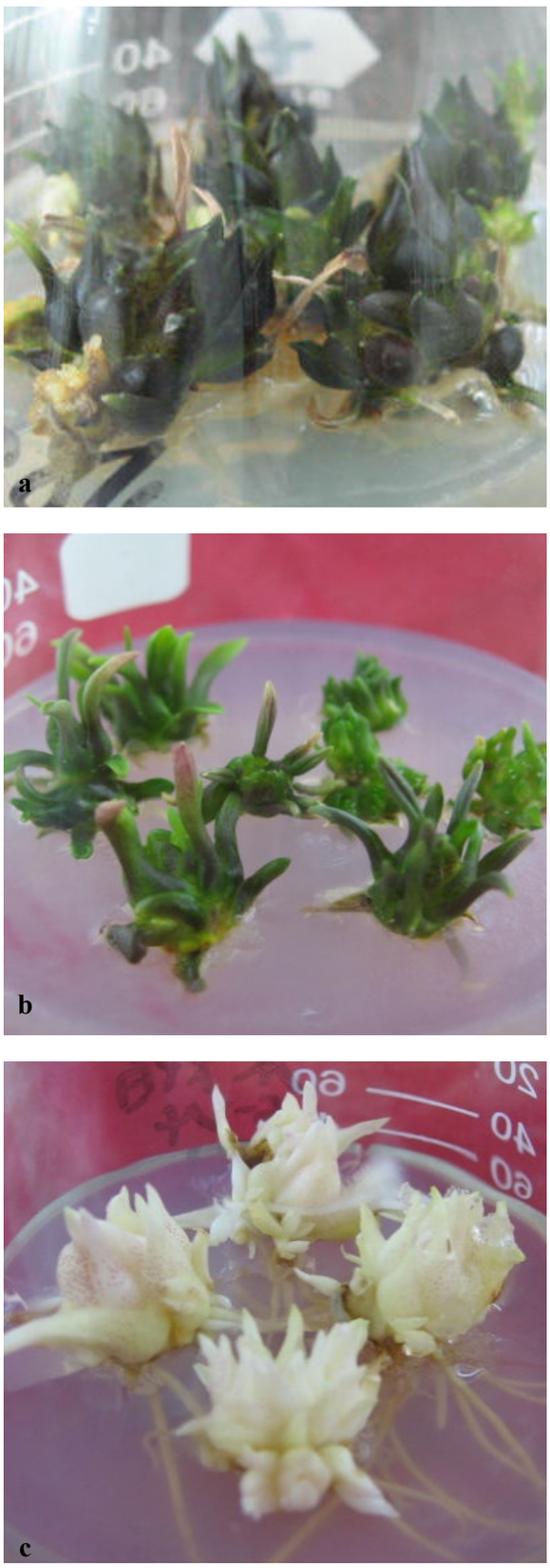

Fig. 2. Bulblets swelling under different light condition
(a): Continuous light; (b): Differentiation under darkness and swelling under light; (c): Continuous light darkness

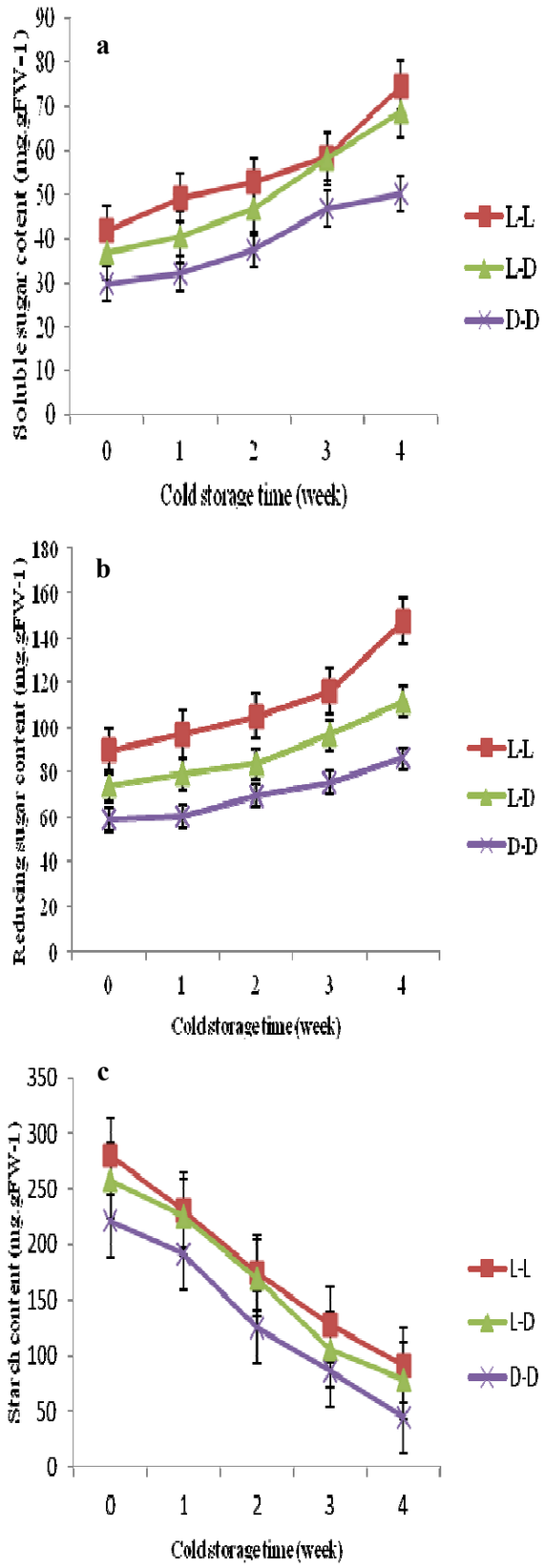

Fig. 3. Carbohydrate content change trends of bulblet cultured under various light conditions during cold storage
(a): Various trends of soluble sugar content; (b): Various trends of reducing sugar content; (c): Various trends of starch content.

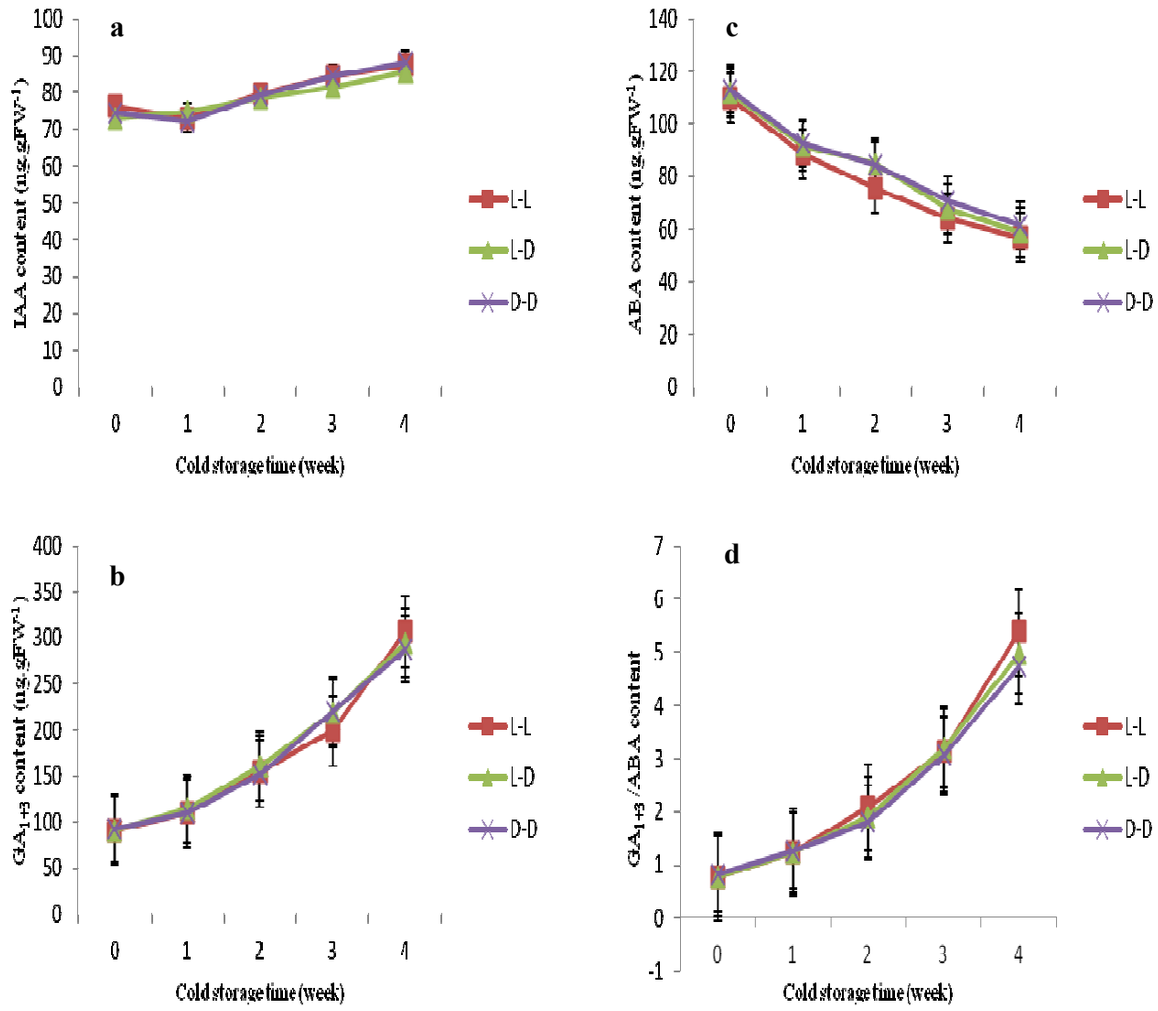

Fig. 4. Different endogenous hormone content change trends of bulblet cultured under various light conditions during cold storage
(a): Various trends of IAA content; (b): Various trends of GA_{1+3} content; (c): Various trends of ABA content; (d) Various trends of GA_{1+3} / ABA content.

In bulblet swelling phase, a higher concentration of sucrose was more effective in increasing average diameter of *In vitro* bulblets confirming the previous studies (De Klerk, 1992; Bonnier & Van Tuyl, 1997; Marinengeli & Curvetto, 1997). The results of the present study revealed that the diameter of bulblet cultured on media with 80 $g.L^{-1}$ of sucrose increased to 350.44% on average. The proliferation multiple (3.11) and roots condition (8.77 roots/bulblet) were quite satisfying (Table 3). Although lower concentration of sucrose could induce more shoots and roots during this phase, it was not optimal for bulb swelling as most of the shoots tended to grow leaves and became plantlet. 90 $g.L^{-1}$ of sucrose made all shoots form bulblet perfectly but restrained the growth of shoots and roots to some extent. It might indicate that sucrose, apart from being the major nutrition content, might play an important role as signal molecule on bulb development for lily.

Effects of light conditions: The effect of light on lily *In vitro* culture has been ambiguous. Results of the previous study implied that high rates of *Lilium* bulblets growth *In vitro* can be obtained by hotoautotrophic conditions (Mei-Lan L *et al.*, 2003). There were also reports showed that light could induce sprouting, as was found for *In vitro* plants of *L. speciosum* (De Klerk & Paffen, 1995). Darkness reduced leaf growth (Takayama & Misawa, 1980). In the present study, it was suggested that light affected differently lily *In vitro* growth during different stages too. Based on our research, a 16-h photoperiod light condition facilitated explants to differentiate. In mean 27.60 days, 83.4% of explants induced 6.4 shoots average per bulb scales (Table 2). More interestingly, 67.36% of the bulb scales cultured in a 16-h photoperiod tended to differentiate adventitious shoots cluster (Fig. 1a). When continuous darkness was applied, 78.69% of explants tended to form bulblet directly with foliage small, leafy bulb scales and abnormally swollen basal plates clasping tightly (Fig. 1b).

When it came to the swelling of bulblet, the bulblet differentiated and swelled in darkness enlarged most (the diameter increase rate was 443.70% on average) with best root condition (14.53 roots per bulblet and average length was 36.82 mm). The worst situation was bulblet differentiated in a 16-h photoperiod and enlarged in darkness (Table 4). At the same time, we noticed that the leaves of bulblet in light were more but smaller in size, dark-green. And the basal portion of bulblet scales induced more shoots easily. The roots were thin, long and high tenacity. However, in darkness, bulblet had fewer but thicker bulblet scales tight together which were white. The roots were thicker, fewer with light yellow color which were fragile and relatively easy to break during transplant.

In vitro regenerated bulblets are reported to exhibit dormancy (Harvey & Selby, 1997; Langens-Gerrits *et al*., 2001), which is controlled by the factors such as temperature, sucrose and ABA (Hole *et al.*, 1989; Aguettaz *et al.*, 1990; Delvallée *et al.*, 1990; Djilianov *et al.*, 1994; Kim *et al.*, 1994; Suttle & Hultstrand, 1994). Langens-Gerrits's (2001) research showed that in 'Snow Queen', light affected the level of dormancy that developed during regeneration at 25 °C. Bulblets regenerated in the light (16-h photoperiod) were less dormant than bulblets regenerated in the dark. In the present study, we found that IAA content of bulblets produced under L-L condition was highest which might indicate that bulblets regenerated in the light (16-h photoperiod) were less dormant than bulblets regenerated in the dark which was consistent with previous study (Langens-Gerrits *et al.*, 2001). In preset research, storage at 4°C for 4 weeks would help to break the dormancy of lily bulblet. In the meantime, content of carbohydrate and endogenous hormones were investigated. Results showed that the carbohydrate and endogenous hormone's change patterns of bulblets-in-tube were similar with commercial Lily bulbs showed the process of their dormancy breaking. The data revealed that the bulblet cultured under L-L condition had most carbohydrate accumulation due to it was photoautotrophic. Following closely up was bulblet in D-D condition with second high of carbohydrate accumulation. The lowest carbohydrate content belonged to the bulblet supplied with L-D condition (Table 5). We conjectured that this might due to growth disorder as light condition changed. There was slight difference on endogenous hormones content which related closely to dormancy breaking among bulblet cultured in different light condition. From Fig. 3a-c and Fig. 4a-d, it was found that the change trends of carbohydrate and different plant growth regulator content had no significant difference between bulblets culturing under different light conditions and similar with lily commercial bulbs. *Ex vitro* performances of those bulblets were also compared. The surviving rate was 66.7% which was also the highest immediately following by bulblet in D-D condition (53.3%). The bulblets in L-D survived least (26.7%). All above suggested that bulblet produced under darkness or at least partial darkness was feasible and had slight difference among them which meant a great deal of energy can be conserved.

Conclusions

In conclusion, we optimized the bulblet-in-tube production protocol. Our results showed that it could be able to direct the growth of explants to meet our different need simply by adjusting the concentration of sucrose accordingly without scanning different type and combination of PRGs. When cultured in 3 $g.L^{-1}$ sucrose concentration, explants and seedlings tended to differentiate and regenerate efficiently, while providing 8 $g.L^{-1}$ concentration of sucrose with no PRGs, seedlings could swell into bulblets satisfyingly. Most of bulblet could germinate after 4 weeks cold storage at 4℃ which was more efficient than result of Han *et al*., (2005). Furthermore, our work also suggested that heterotrophic strategy was possible which are therefore recommended for the production of high-quality bulblets-in-tube especially during the long time bulblet swelling phase. This new protocol of was quite beneficial for the massive bulblet-in-tube production and more importantly, had important significance for energy conservation and cost reduction.

Acknowledgments

This research was financially supported by the National Natural Science Fund (31071819), the planning subject of 'the twelfth five-year-plan' in national science and technology for the rural development in China (2011BAD12B02) and Beijing Municipal Bureau of landscaping program (YLHH201200116), which are gratefully acknowledged.

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(Received for publication 10 October 2012)