Electronic Journal of Biotechnology ISSN: 0717-3458
Vol.10 No.4, Issue of October 15, 2007

Received February 27, 2006 / Accepted April 18, 2007
DOI: 10.2225/vol10-issue4-fulltext-7
RESEARCH ARTICLE

Micropropagation of *Lilium ledebourii* (Baker) Boiss as affected by plant growth regulator, sucrose concentration, harvesting season and cold treatments

Pejman Azadi
Tissue Culture Department
National Research Center of Ornamental Plants
P.O. Box: 37815-137, Mahallat, Iran
Tel: 988663227393
Fax: 988663229394
E-mail: azadip22@yahoo.com

Morteza Khosh-Khui*
Department of Horticultural Science
College of Agriculture
Shiraz University
Shiraz, Iran
Tel: 987112287103
Fax: 987116289017
E-mail: mkhoshkhui@yahoo.com

Keywords: bulblet, cold treatment, conservation, harvesting season, *Lilium ledebourii*, tissue culture.

Abbreviations: BA: benzyladenin
IAA: Indole-3-acetic acid
MS: Murashige and Skoog
NAA: naphthaleneacetic acid

A protocol for the micropropagation in different harvesting time of *Lilium ledebourii* (Baker) Boiss, an endangered rare species endemic to Iran has been developed. *In vitro* scale culture of this species, using bulbs from three harvesting seasons (spring, summer and winter), was attempted. Among the various treatments tested, the Murashige and Skoog (MS) medium supplemented with 0.1 mg l^{-1} naphthaleneacetic acid (NAA) + 0.1 mg l^{-1} benzyladenin (BA) and 6% sucrose in all harvesting seasons proved to be superior to others. The best results for fresh weight of bulblets, rooting parameters and the survival rate after transplantation to greenhouse were obtained from early winter-harvested bulbs. Summer-harvested bulbs had the highest number of bulblets per explant. The bulblets at the end of the culture period were given cold treatment at 4ºC for 2-8 weeks at a 2-weeks interval and then transplanted to a potting mixture of sand, leaf mold and peat moss (1:1:1 v/v). The best emergence rate (90%) was achieved at 8 weeks cold treatment for winter harvested bulbs.

Lilium ledebourii (Baker) Boiss (Liliaceae) is a perennial endangered rare species endemic to Iran (Figure 1). This plant grows at altitudes between 1750-2100 m in Euxino-Hyrcanian province and it is under surveillance of Iranian Regional Environmental Protection Agency (Jalili and Jamzad, 1999). *L. ledebourii* has high ornamental value for the beautiful white flowers and a large, raceme having 2-15 flowers that can be used for breeding programs. The number of plants of this species is continuously decreasing in nature because of cutting the plants and removing underground organs. The application of *in vitro* propagation techniques may offer the possibility of producing large number of uniform plants for breeding programs and further field culture.

The successful use of tissue culture techniques for rapid propagation of some species of the genus *Lilium* has been reported including *L. longiflorum* (Tanimoto and Matsubara, 1995; Arzate-Fernandez et al. 1997; Nhut, 1998; Nhut et al. 2001; Nhut et al. 2002; Bacchetta et al. 2003; Nhut, 2003), *L. japonicum* (Yamagishi, 1998), *L. speciosum* (Chang et al. 2000), *L. concolor* (Jeong, 1996), *L. nepalense* (Wawrosch et al. 2001), *Lilium regale* Wil (Pelkonen and Kauppi, 1999), *Lilium* oriental hybrid (Lian et al. 2002a), *Lilium* Asiatic hybrid (Lian et al. 2003).

One of the best and most prolific vegetative propagation method for lilies is *in vitro* scale culture (Jeong, 1996; Varshney et al. 2001; Bahr and Compton, 2004). *In vitro* adventitious bud regeneration from scales of *Lilium* depends on factors such as auxin and cytokinin

*Corresponding author

concentrations (Jeong, 1996; Varshney et al. 2000), sucrose concentration (Jeong, 1996; Varshney et al. 2000; Kumar et al. 2005), light treatment (Varshney et al. 2000; Lian et al. 2002b; Kumar et al. 2005), scale position or kind of explant (Jeong, 1996; Varshney et al. 2000). There are no reports on micropropagation of *L. ledebourii*. This paper introduces a protocol for the *in vitro* propagation and reports the suitable season for harvesting the valuable bulbs of this species, with best cold treatment for sprouting of bulblets.

MATERIALS AND METHODS

Plant material and explant source

Fresh bulbs of *Lilium ledebourii* were collected from Damash of Emarlu in the Alborz range of mountains in three seasons: spring (May 2002), summer (July 2002) and winter (January 2003), and then stored at 4ºC for one month. The middle scales of bulbs were separated and washed thoroughly under running tap water. Following a 5 sec treatment with 70% ethanol, then surface sterilized in a 2% sodium hypochlorite solution (NaClO) for 30 min and rinsed three times in sterilized distilled water. Basal segments of the scales measuring about 1 x 1 cm^2 were aseptically cut and each segment with the dorsal side in contact with the medium was placed in a 22 x 180 mm test tube containing 15 ml of the culture medium.

Medium and culture conditions

Murashige and Skoog (1962) media without hormones or supplemented with naphthaleneacetic acid (NAA) (0.1 mg l^{-1}) + benzyladenin (BA) (0.1 mg l^{-1}) and NAA (0.5 mg l^{-1}) + BA (0.5 mg l^{-1}) (Sigma, St. Louis, U.S.A.) were used. Each was combined factorially with sucrose (3, 6 and 9%). The media were solidified with 7 g l^{-1} Merck agar-agar. The pH of media were adjusted to 5.7 before dispensing in test tubes and autoclaving at 121ºC and 1.5 kg cm^{-1} pressure for 20 min. The cultures were kept for 16 weeks at 25 ± 1ºC under a 16 hrs photoperiod at a light intensity of 45 µ mol m^{-2} s^{-1} emitted by cool-white fluorescent tubes.

Figure 1. *Lilium ledebourii* (Baker) Boiss (Liliaceae) a perennial endangered rare species that grows at altitudes between 1750-2100 m in Euxino-Hyrcanian province and it is under surveillance of Iranian Regional Environmental Protection Agency.

Cold treatment and transplantation

As it was reported that *Lilium* bulblet weight affected the sprouting (Langens-Gerrits et al. 2003), only bulblets with 60-100 mg in weight were used for cold treatment experiment. The bulblets were cold treated at 4ºC for 0, 2, 4, 6 and 8 weeks in all three harvesting seasons, then transplanted in pots containing sand, leaf-mold and peat moss (1:1:1 v/v) and placed in a greenhouse with 22 ± 2ºC mean temperature, 70 ± 5% relative humidity and natural light conditions.

Data collection and statistical analysis

For scale cultures from the bulbs harvested in the three

Table 1. Effects of growth regulators combined with three concentrations of sucrose on bulblet regeneration and rooting parameters of *Lilium ledebourii* in spring harvested bulbs.

NAA + BA (mg l-1)	Sucrose (%)	Bulblet formation (%)	No. of bulblets per scale	Mean fresh wt (weight) of bulblet (mg)	Rooting (%)	No. of roots per bulblet	Root length (mm)
0	3	58.3 aa	2.25 cd	41.5 d	57.0 c	1.71 ab	7.67 cd
	6	69.5 a	4.27 ab	43.2 d	71.4 abc	1.55 ab	9.72 bcd
	9	25.0 b	0.98 d	59.6 cd	26.7 d	1.00 b	7.00 cd
0.1 + 0.1	3	80.6 a	5.41 a	158.3 abc	60.9 bc	2.17 ab	10.37 bcd
	6	63.9 a	3.11 bc	236.8 a	87.3 a	2.89 a	17.32 ab
	9	33.3 b	1.75 cd	83.5 bcd	80.9 ab	2.73 a	10.96 bcd
0.5 + 0.5	3	83.3 a	2.92 bc	128.5 abcd	75.0 abc	1.10 a	5.99 d
	6	61.1 a	3.67 b	113.6 bcd	84.0 ab	2.52 a	15.09 abc
	9	25.0 b	1.25 d	177.1 ab	77.0 abc	2.73 a	20.82 a

Mean values followed by the same letters are not significantly different at the 5% level (Duncan's multiple range test).

Figure 2. Effects of plant growth regulator and sucrose concentrations on bulblet formation of *Lilium ledebourii* (Baker) Boiss on MS + BA (0.1 mg l^{-1}) + NAA (0.1 mg l^{-1}) and 3% sucrose in spring-harvested bulbs, after 16 weeks of culture.

seasons, the percentage of bulblet formation, mean number of bulblets per explant, fresh weight of bulblets, percentage of rooting, number of roots per bulblets and length of roots were recorded after a 16-week period without subculture. The experiment was conducted in factorial on the basis of completely randomized design with three replications. Each replication consisted of 12-16 test tubes each with one explant. For mean comparisons Duncan's new multiple range tests were used.

RESULTS

Effect of growth regulator and sucrose concentrations on bulblet regeneration in spring-harvested bulbs

The highest percentage of bulblet formation (83.3%) was observed on the medium containing 0.5 mg l^{-1} BA + 0.5 mg l^{-1} NAA and 3% sucrose (Table 1). The highest number of bulblets (5.41) was obtained on the medium containing 0.1 mg l^{-1} BA + 0.1 mg l^{-1} NAA and 3% sucrose (Figure 2). It was also noted that high concentration of sucrose (9%) decreased the number of bulblets per explant. The presence of growth regulators significantly increased the growth of bulblets compared with control. The fresh weight of bulblets was greatest (237 mg) in 0.1 mg l^{-1} BA + 0.1 mg l^{-1} NAA and 6% sucrose. For rooting parameters the best result was achieved on the medium containing 0.1 mg l^{-1} BA + 0.1 mg l^{-1} NAA and 6% sucrose (Table 1).

Effect of growth regulator and sucrose concentrations on bulblet regeneration in summer-harvested bulbs

As it is evident from Table 2, NAA and BA did not significantly affect the percentage of bulblet formation while the effect of sucrose was significant. All the media with 3% sucrose resulted in 100% bulblet formation. Similarly, the medium containing 0.1 mg l^{-1} BA + 0.1 mg l^{-1} NAA and 6% sucrose resulted in 100% bulblet formation. The highest number of bulblets (14.8) was achieved with the same medium. Combination of 0.5 mg l^{-1} BA + 0.5 mg l^{-1} NAA with 6% sucrose led to the greatest fresh weight of bulblets (74.3 mg) (Table 2 and Figure 3). The percentage of rooting, root number and root length increased by increasing sucrose concentration from 3% to 6% for all media. The effects of NAA and BA were insignificant for all rooting parameters (Table 2).

Effect of growth regulator and sucrose concentrations on bulblet regeneration in winter-harvested bulbs

The effect of growth regulators and sucrose did not influence the percentage of bulblet formation and most treatments resulted in 100% bulblet formation (Table 3). The highest number of bulblets was obtained on the

Table 2. Effect of growth regulators combined with three concentrations of sucrose on bulblet regeneration and rooting parameters of *Lilium ledebourii* in summer harvested bulbs.

NAA + BA (mg l-1)	Sucrose (%)	Bulblet formation (%)	No. of bulblets per scale	Mean fresh wt (weight) of bulblet (mg)	Rooting (%)	No. of roots per bulblet	Root length (mm)
0	3	100 aa	6.56 bc	51.9 ab	36.9 bcd	0.79 bc	6.09 bc
	6	75 ab	3.83 cd	58.4 ab	58.9 ab	1.41 ab	8.15 abc
	9	72.2 ab	2.50 d	29.7 b	53.2 abc	1.73 a	5.70 bc
0.1 + 0.1	3	100 a	9.11 b	34.8 b	21.2 d	0.25 c	2.29 c
	6	100 a	14.81 a	50.0 ab	60.8 a	1.29 ab	11.41 ab
	9	72.2 b	7.53 b	66.4 a	55.9 ab	1.84 a	7.17 abc
0.5 + 0.5	3	100 a	9.28 b	47.5 ab	31.9 cd	0.45 c	3.33 c
	6	66.7 b	6.75 bc	74.3 a	67.8 a	1.82 a	12.93 a
	9	63.9 b	6.22 bc	65.5 a	60.4 a	0.88 bc	7.01 abc

[a]Mean values followed by the same letters are not significantly different at the 5% level (Duncan's multiple range test).

Figure 3. Effects of MS medium + BA (0.5 mgl^{-1}) + NAA (0.5 mgl^{-1}) combined with 3% (a), 6% (b), or 9% (c) sucrose concentrations on micropropagation of *Lilium ledebourii* (Baker) Boiss in summer-harvested bulbs, after 16 weeks of culture.

medium with 0.1 mg l^{-1} BA + 0.1 mg l^{-1} NAA and 9% sucrose. Number of bulblets per scale decreased with adding 0.5 mg l^{-1} BA + 0.5 mg l^{-1} NAA, but increased with increasing the sucrose concentration. The presence of growth regulators increased fresh weight of bulblets compared with control. The combinations of 0.1 mg l^{-1} BA + 0.1 mg l^{-1} NAA supplemented with 3% and 6% sucrose led to a maximum fresh weight of bulblets (385.8 and 306.9 mg, respectively). However, the addition of higher concentration of sucrose (9%) caused a decrease in bulblet growth. The effects of sucrose on rooting parameters were significant. The addition of 6% and 9% sucrose increased the percentage of rooting, root number and root length, but the effect of growth regulators were not significant (Table 3).

Comparison of the effect of plant growth regulator and sucrose concentrations on means of three harvesting seasons

The comparison among three harvesting seasons is presented in Figure 4. The effects of growth regulators were not significant on the percentage of bulblet formation for bulbs harvested in the three seasons. The effect of sucrose was significant in spring and summer *i.e.* the percentage of bulblet formation was significantly reduced by increasing the concentration of sucrose but it was insignificant in winter-harvested bulbs. The effects of NAA and BA were significant for the number of bulblets in all three seasons. The highest number of bulblets was formed on media with 0.1 mg l^{-1} NAA + 0.1 mg l^{-1} BA in summer. In spring and summer seasons, high concentration (0.5 mg l^{-1} NAA + 0.5 mg l^{-1} BA) or the absence of growth regulators significantly decreased the number of bulblets, while in winter the number of bulblets was reduced only at high concentration of growth regulators (0.5 mg l^{-1} NAA + 0.5 mg l^{-1} BA). The effect of sucrose was similar in spring and summer with 3% and 6% sucrose concentration, whereas the highest sucrose concentration (9%) significantly reduced the number of bulblets in both seasons. However, this concentration increased the number of bulblets in the winter. The average number of bulblets was highest in summer and lowest in spring. The fresh weight of bulblets in summer was poor and the addition of NAA and BA was not effective on this trait. Sucrose concentration of 3 or 6% was optimum for increasing the fresh weight of bulblets for winter. The results showed that in all treatments the highest and lowest fresh weight of bulblets belonged to winter and summer-harvested bulbs, respectively (Figure 4).

The effects of NAA and BA on percentage of root formation were significant only for spring-harvested bulbs. Just in this season, the addition of exogenous plant growth hormones increased rooting parameters. The effect of sucrose was significant for all rooting parameters in the three seasons except for the root number in spring. For all seasons, 6% sucrose was the best concentration for rooting parameters. Percentage of bulblet rooting, number of roots

Table 3. Effect of growth regulators combined with three concentrations of sucrose on bulblet regenerations and rooting parameters of *Lilium ledebourii* in winter harvested bulbs.

NAA + BA (mg l-1)	Sucrose (%)	Bulblet Formation (%)	No. of bulblets per scale	Mean fresh wt (weight) of bulblet (mg)	Rooting (%)	No. of roots per bulblet	Root l ength (mm)
0	3	91.7 aba	4.92 abc	120.8 b	62.5 bc	2.01 ab	10.98 bc
	6	100 a	5.08 abc	185.2 ab	96.6 a	3.85 a	19.28 ab
	9	100 a	5.94 ab	127.3 b	78.1 abc	3.09 ab	17.68 ab
0.1 + 0.1	3	100 a	3.83 bc	385.8 a	73.1 abc	2.98 ab	12.39 bc
	6	100 a	5.03 abc	306.9 ab	90.6 ab	3.96 a	23.64 a
	9	100 a	6.89 a	135.9 b	85.5 ab	2.53 ab	22.61 a
0.5 + 0.5	3	100 a	3.22 c	178.6 ab	50.0 c	1.21 b	7.36 c
	6	100 a	4.25 bc	200.1 ab	88.2 ab	3.32 ab	24.27 a
	9	80.6 b	4.03 bc	203.3 ab	85.2 ab	3.02 ab	23.14 a

[a]Mean values followed by the same letters are not significantly different at the 5% level (Duncan's multiple range test).

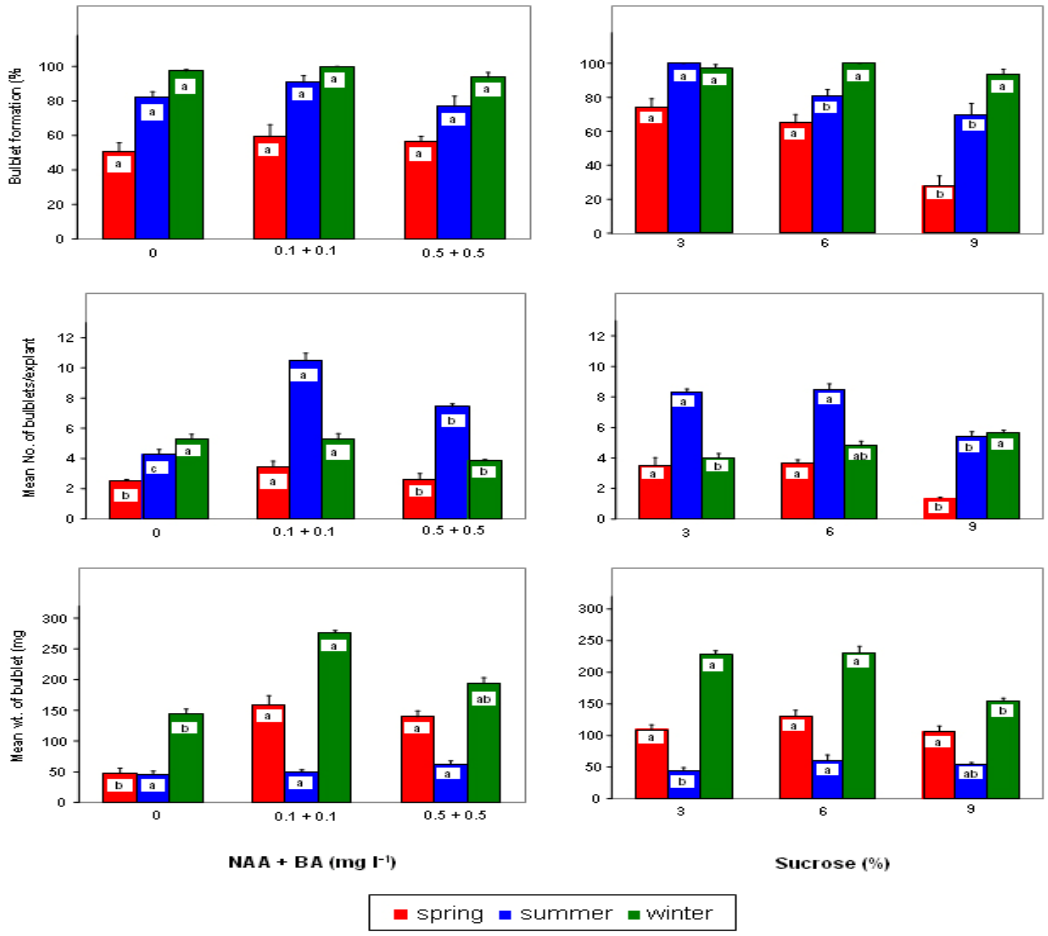

Figure 4. Effects of growth regulator and sucrose concentrations on percentages of bulblet formation, mean number of bulblets per explant and fresh weight (wt) of bulblet in spring, summer and winter-harvested bulbs of *Lilium ledebourii*. Bars with the same letters are not significantly different at the 5% level of probability using DNMRT. Vertical bars indicate standard error.

and length of roots were highest in the winter and lowest in the summer (Figure 5).

Cold treatment and transplantation

Increasing the period of cold treatment from 2 to 8 weeks increased the emergence of bulblets at three harvesting seasons. The bulblets were sprouted after 2 months (Figure 6). The survival rate of bulblets cold treated for 8 weeks (the best treatment) after 5 months was 80%, 65% and 90% for spring, summer and winter-harvested bulbs, respectively. The horticultural habits of the cloning plants are under investigation to introduce the germplasm for a lily breeding program.

DISCUSSION

Percentage of bulblet formation

In agreement with Niimi (1985) who reported that the addition of BA and NAA had no significant effect on bulblets formation in *L. rubellum*, the data of present study showed that the addition of NAA and BA did not significantly affect the percentage of bulblet formation in *L. ledebourii*. Similarly, results of this investigation for all seasons are in accordance with Jeong (1996) findings who showed that the highest percentage of bulblets was obtained on a medium with 3% sucrose in *L. concolor*. Dantu and Bhojwani (1995) showed that in *Gladiolus* the percentage of cormlet formation was increased using 6% sucrose in the medium but further increases in sucrose concentration decreased the percentage of cormlet formation. In the present investigation, the percentage of bulblet formation was reduced by increasing the concentration of sucrose in bulbs harvested in spring and summer.

Robb (1957) working with *Lilium speciosum* reported that explants removed during summer or winter months hardly produced bulblets. In contrast, data of present study showed that in *L. ledebourii* the average of the highest percentage of the bulblet formation was achieved on bulbs harvested in winter and summer. A remarkable influence of the season

on tissue culture response of barley (*Hordeum vulgare*) for cultivars 'Salome' and 'Golden Promise' was observed. Scutella from immature embryos of cv. 'Salome' showed an increase in the frequency of plant regeneration from January to June, reaching the highest values in March/April followed by a decrease in May and a continuous and strong decrease from June to December. The same seasonal effect was evident for plant regeneration from scutella of cv. 'Golden Promise'. In contrast to plant regeneration frequency, the percentage of embryogenic callus formation showed different result for 'Salome', and a curve with a slight optimum in March for the first six months was observed, but in the second half of the year there were also high values visible. In case of embryogenic callus formation from 'Golden Promise', a relatively uniform reaction in the first six months of the year followed by a pronounced decrease in November was observed (Sharma et al. 2005). Another observations regarding the seasonal variation in regeneration frequency from barley microspores were provided by Ritala et al. (2001), they obtained the highest values, when spikes were harvested from May to December, whereas lowest responsibility was detected for the harvesting time from January up to April. These results show that genetic variation is responsible for such controversy.

Due to the strong seasonal differences in the sun height, the way of the light passed through the atmosphere differs leading to a different spectral composition of global irradiance. Thus in winter time and the months close to winter, natural light contains more red light, the short waved parts were filtered out during the long way through the atmosphere. Various studies have shown correlations between light response and changes in auxin levels (reviewed in Tian and Reed, 2001). For example, the red light decreased the auxin levels in maize coleoptile tips and mesocotyls (Barker-Bridgers et al. 1998). Considering that the high embryogenic competence in maize and wheat immature embryos is correlated with a low Indole-3-acetic acid (IAA) content (Carnes and Wright, 1988; Hess and Carman, 1998), it could be possible that the higher embryogenic reactivity of barley observed between

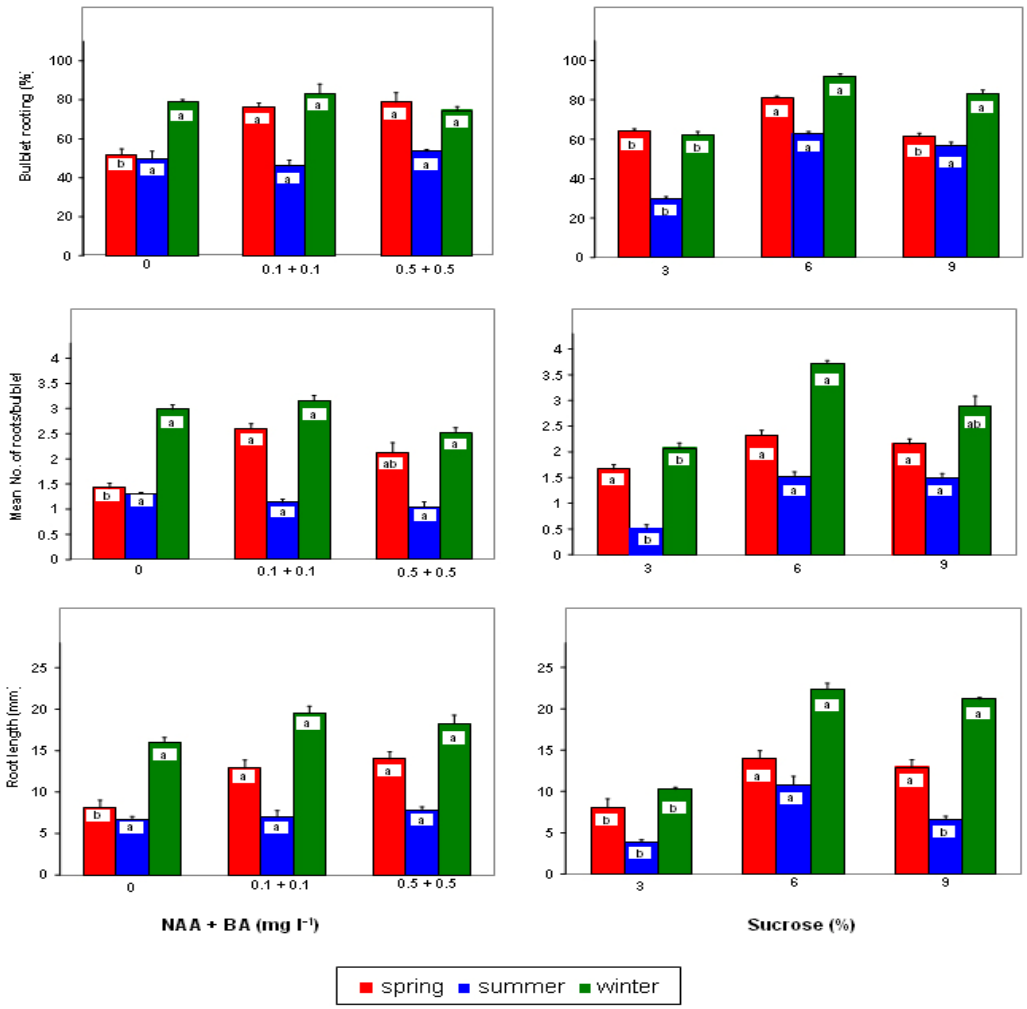

Figure 5. Effects of growth regulator and sucrose concentrations on percentages of rooting, mean number of root and root length in spring, summer and winter-harvested bulbs of *Lilium ledebourii*. Bars with the same letters are not significantly different

February and June is connected with the higher amount of red light at that time. This hypothesis is consistent with observations of a promoting influence of red light on embryogenic callus formation whereas white to blue light treatments were inhibitory to somatic embryogenesis in different plant species (Karnachuk and Gvozdeva, 1998; Bach and Krol, 2001). Bulbs of *Lilium longiflorum* Thunb exposed to red or far red light for 30 days prior to planting emerged much faster than bulbs not exposed to light (Miller, 1993). Different responses in tissue culture of *Lilium* might be caused by environmental parameters such as light quality during growth seasons.

Number of bulblets per scale

Niimi (1985) reported that the addition of 0.1 mg l^{-1} BA + 0.1 mg l^{-1} NAA had no significant effect on the number of bulblets from bulb scale culturein *L. rubellum*. Whereas, Jeong (1996) indicated that the addition of the hormones (more than 0.1 mg l^{-1} NAA with or without BA) decreased the number of bulblets in Korean lilies bulb scale culture.The present study indicated that in the species under study the addition of low concentration of BA and NAA was required to increase the number of bulblets for spring and summer but not necessary for winter-harvested bulblets. In *L. speciosum in vitro* proliferation occurred only during periods when the plants were in a vegetative condition (Robb, 1957). In this investigation, it was shown that *in vitro* proliferation in *L. ledebourii* could occur even in winter.

Endogenous hormones may be one of most important factors determining the bulblet production of explants besides the genetic background. The crucial effect of endogenous hormone and donor plant environment for embryogenesis were shown by Carnes and Wright (1988) and Hess and Carman (1998). The seasonal variation of endogenous plant growth hormones in the cambial region of *Pinus contorta* (Savidge et al. 1982) and bulb of *Lilium* Oriental Hybrid 'Casa Blanca' (Kim and Kim, 2005) were reported.

Hong-Mei et al. (2005) reported that with increasing the storage time of bulbs of *Lilium davidii* var. unicolour kept under cold treatment the contents of starch and ABA decreased markedly but the level of total soluble sugar, IAA and GA3 increased sharply. Because of natural condition for bulbs of *Lilium ledebourii* during late autumn and winter time (when the bulbs are covered by snow), this natural cold treatment could be effective to increase the level of endogenous hormones. Kim and Kim (2005) reported that IAA content increased in scales during bulb maturation. IAA content in scale of *Lilium* Oriental Hybrid 'Casa Blanca' was high at 60 days after flowering. In this study the number of bulblet of summer-harvested bulb (about 45 days after flowering), cultured on a media without hormones and supplemented with 3% sucrose, was 2.92 and 1.33 folds more than spring and winter-harvested bulbs, respectively (Table 1, Table 2 and Table 3).

Varshney et al. (2000) reported that 6% and 9% sucrose were considered optimal for a high multiplication rate in cultivars of lily Asiatic hybrids 'Gran Paradiso' and 'Sanciro', respectively. Further increase in sucrose concentration decreased the number of bulblets for both cultivars. According to Kumar et al. (2005), the number of bulblets was higher at 9% than at 6% sucrose in Oriental hybrid lily 'Star Gazer'. Data obtained for *L. ledebourii* showed that high concentration of sucrose (9%) increased the number of bulblets for winter-harvested bulbs, principally with 0.1 mg l^{-1} NAA + 0.1 mg l^{-1} BA. The starch concentration in *L. longiflorum* organs was highly variable and highly dependent on environmental conditions. During shoot growth, starch content decreased while sucrose concentration increased (Miller, 1993). As the concentration of sucrose during spring and summer seasons is high in scales, increasing of sucrose (more than 3%) was not effective in this experiment (Figure 4).

Figure 6. Growth of bulblets of *Lilium ledebourii* (Baker) Boiss from winter harvested bulbs after 2 months being grown in the greenhouse.

Fresh weight of bulblets

Niimi (1985) reported that addition of NAA (0.1 or 1 mg l^{-1}) and BA (0.1 mg l^{-1}) significantly increased the fresh weight of bulblets in *L. rubellum*. During shoot growth, starch content decreased and scales were depleted of dry weight at anthesis, while cold treatment increased total bulb protein concentration by 50% and the amino acid concentration was doubled (Miller, 1993). In the present study, fresh weight of bulblet in winter-harvested bulb at medium without hormones and supplemented with 3% sucrose was 2.91 and 2.32 folds more than summer and spring-harvested bulbs, respectively (Table 1, Table 2 and

Table 3). The data reported here similarly showed that addition of BA and NAA was essential to increase fresh weight of bulblet in bulbs harvested in spring and winter. However, they were not effective on fresh weight of bulblet in summer (Figure 4). It seems that increase in fresh weight of bulblet not only depends on endogenous hormones but also depends on protein content of bulb scale.

Rooting parameters

Wawrosch et al. (2001) reported that the absence of exogenous hormones did not encourage root formation in *L. nepalense*. However, in *L. longiflorum* it was reported that the addition of NAA significantly increased root numbers in comparison to hormone-free medium (Nhut, 1998). In this study the addition of hormones was insignificant in bulbs harvested in winter and summer. However, it was effective for increasing of rooting parameters in spring. All rooting parameters decreased from winter to summer, root number at medium without hormones and supplemented with 3% sucrose in winter-harvested bulb was 1.18 and 2.66 folds more than spring and summer-harvested bulb, respectively (Table 1, Table 2 and Table 3). It might be due to increase in IAA content after cold treatment (Hong-Mei et al. 2005).

Takayama and Misawa (1979) reported that the optimum level of sucrose for bulblet formation was lower than for root formation in *Lilium speciosum* and *L. auratum*. The data obtained in this experiment for all seasons is in agreement with their results. The addition of 6% sucrose increased significantly the percentage of rooting, root number and root length for all seasons, but no increasing or low increasing was observed for bulblet formation parameters including the percentage of bulblet formation, number of bulblet and weight of bulblet (Figure 4 and Figure 5).

Cold treatment

Previous report on the sprouting of bulblets indicated that prolonging the cold treatment before transfer to the greenhouse improved plant development (Bahr and Compton, 2004). Data of present study showed that the best emergence rate of *L. ledebourii* was achieved at 8 weeks cold (4ºC) treatment in all the three harvesting seasons. This is in agreement with De Klerk et al. (1992) who reported that the percentage of sprouted bulblets increased with the length of cold treatment.

CONCLUDING REMARKS

Based on the present study, the best harvesting season for micropropagation of *L. ledebourii* is early winter. MS medium supplemented with 0.1 mg l^{-1} NAA + 0.1 mg l^{-1} BA and 6% sucrose was optimal for all the parameters, especially for the weight of bulblets which is a very important factor for the acclimatization stage. The bulblets of winter-harvesting time had the best survival rate in comparison with the two other harvesting seasons. This study demonstrated that the harvesting seasons were very effective on quantity and quality of the regeneration responses.

ACKNOWLEDGMENTS

The authors wish to thank Dr. M.J. Kermani, Dr H. Hashemi and Dr B. Ghareyazie for critical reading of the manuscript and their valuable suggestions and also Ms. A. Safari for her useful assistance.

REFERENCES

ARZATE-FERNANDEZ, A.-M.; NAKAZAKI, T.; OKUMOTO, Y. and TANISAKA, T. Efficient callus induction and plant regeneration from filaments with anther in lily (*Lilium longiflorum* Thunb.). *Plant Cell Reports*, October 1997, vol. 16, no. 12, p. 836-840.

BACCHETTA, Loretta; REMOTTI, Patrizio C; BERNARDINI, Claudia and SACCARDO, Francesco. Adventitious shoot regeneration from leaf explants and stem nodes of *Lilium*. *Plant Cell, Tissue and Organ Culture*, July 2003, vol. 74, no. 1, p. 37-44.

BACH, A. and KROL, A. Effect of light quality on somatic embryogenesis in *Hyacinthus orientalis* L. 'Delft's blue'. *Biological Bulletin of Poznan*, 2001, vol. 38, p. 103-107.

BAHR, Laura R. and COMPTON, Michael E. Competence for *in vitro* bulblet regeneration among eight *Lilium* genotypes. *HortScience*, February 2004, vol. 39, no. 1, p. 127-129.

BARKER-BRIDGERS, Michele; RIBNICKY, David M.; COHEN, Jerry D. and JONES, Alan M. Red-light-regulated growth: Changes in the abundance of indoleacetic acid in the maize (*Zea mays* L.) mesocotyl. *Planta*, January 1998, vol. 204, no. 2, p. 207-211.

CARNES, Michael G. and WRIGHT, Martha S. Endogenous hormone levels of immature corn kernels of A188, Missouri-17 and Dekalb XL-12. *Plant Science*, 1988, vol. 57, no. 3, p. 195-203.

CHANG, Chen; CHEN, Chang-Tesrn; TSAI, Yu-Ching and CHANG, Wei-Chin. A tissue culture protocol for propagation of a rare plant, *Lilium speciosum* Thunb. var. glorisoides Baker. *Botanical Bulletin of Academia Sinica*, April 2000, vol. 41, no. 2, p. 139-142.

DANTU, Prem K. and BHOJWANI, Sant S. *In vitro* corm formation and field evaluation of corm-derived plants of

Gladiolus. *Scientia Horticulturae*, February 1995, vol. 61, no. 1-2, p. 115-129.

DE KLERK, Geert-Jan; DELVALLEE, Isabelle and PAFFEN, Annie. Dormancy release of micropropagated bulblets of *Lilium speciosum* after long culture in soil. *HortScience*, 1992, vol. 27, no. 2, p. 147-148.

HESS, J. Richard and CARMAN, John G. Embryogenic competence of immature wheat embryos: Genotype, donor plant environment, and endogenous hormone levels. *Crop Science*, January-February 1998, vol. 38, no. 1, p. 249-253.

HONG-MEI, Sun; TIAN-LAI, Li and YUN-FEI, Li. Physiology mechanism of metabolisms in the middle scales of *Lilium davidii* var. unicolor bulbs stored at low temperature for dormancy-release. *Agricultural Sciences in China*, 2005, vol. 4, no. 7, p. 521-527.

JALILI, Adel and JAMZAD, Ziba. *Red Data Book of Iran.* Research Institute of Forest and Rangelands, Tehran, 1999. 748 p. ISBN 964-473-061-5.

JEONG, J.H. *In vitro* propagation of bulb scale section of several Korean native lilies. *Acta Horticulturae*, 1996, vol. 414, p. 269-276.

KARNACHUK, R.A. and GVOZDEVA, E.S. Effect of light on phytohormone balance and morphogenesis in wheat embryogenic tissue culture. *Fiziologiya Rastenii*, March 1998, vol. 45, no. 2, p. 289-295.

KIM, K.J. and KIM, K.S. Changes of endogenous growth substances during bulb maturation after flowering in *Lilium* Oriental Hybrid 'Casa Blanca'. *Acta Horticulturae*, 2005, vol. 673, p. 661-665.

KUMAR, S.; KASHYAP, M. and SHARMA, D.R. *In vitro* regeneration and bulblet growth from lily bulb scale explants as affected by retardants, sucrose and irradiance. *Biologia Plantarum*, December 2005, vol. 49, no. 4, p. 629-632.

LANGENS-GERRITS, Merel; DE KLERK, Geert-Jan and CROES, Anton. Phase change in lily bulblets regenerated *in vitro*. *Physiologia Plantarum*, December 2003, vol. 119, no. 4, p. 590-597.

LIAN, M.; CHAKRABARTY, D. and PAEK, K.Y. Growth and uptake of sucrose and mineral ions by bulblets of *Lilium* oriental hybrid 'Casablanca' during bioreactor culture. *The Journal of Horticultural Science and Biotechnology*, May 2002a, vol. 77, no. 3, p. 253-257.

LIAN, M.-L.; MURTHY, H.N and PAEK, K.-Y. Effects of light emitting diodes (LEDs) on the *in vitro* induction and growth of bulblets of *Lilium* oriental hybrid 'Pesaro'. *Scientia Horticulturae*, June 2002b. vol. 94, no. 3, p. 365-370.

LIAN, M.-L.; MURTHY, H.N and PAEK, K.-Y. Photoautotrophic culture conditions and photosynthetic photon flux influence growth of *Lilium* bulblets *in vitro*. *In Vitro Cellular and Development Biology-Plant*, September-October 2003, vol. 39, no. 5, p. 532-535.

MILLER, William B. *Lilium longiflorum*. In: DE HERTOGH, August and LE NARD, Marcel eds. *The Physiology of Flower Bulbs*. Amsterdam, Elsevier Science Publishing, 1993, p. 391-422.

MURASHIGE, T. and SKOOG, F. A revised medium for rapid growth and bioassays with tobacco tissue culture. *Physiologia Plantarum*, 1962, vol. 15, p. 473-497.

NHUT, Duong Tan. Micropropagation of lily (*Lilium longiflorum*) via *in vitro* stem node and pseudo-bulblet culture. *Plant Cell Reports*, September 1998, vol. 17, no. 12, p. 913-916.

NHUT, Duong Tan; LE, Bui V.; FUKAI, Seiichi; TANAKA, Michio and THANH, Van K.T. Effects of activated charcoal, explant size, explant position and sucrose concentration on plant and shoot regeneration of *Lilium longiflorum* via young stem culture. *Plant Growth Regulation*, January 2001, vol. 33, no. 1, p. 59-65.

NHUT, Duong Tan; HUONG, Nguyen Thi Dieu; LE, Bui Van; DA SILVA, Jaime T.; FUKAI, Seiichi and TANAKA, Michio. The changes in shoot regeneration potential of protocorm-like bodies derived from *Lilium Iongiflorum* young stem explants exposed to medium volume, pH, light intensity and sucrose concentration pretreatment. *The Journal of Horticultural Science and Biotechnology*, January 2002, vol. 77, no. 1, p. 79-82.

NHUT, Duong Tan. The control of *in vitro* direct main stem formation of *Lilium longiflorum* derived from receptacle culture and rapid propagation by using *in vitro* stem nodes. *Plant Growth Regulation*, June 2003, vol. 40, no. 2, p. 179-184.

NIIMI, Yoshiji. Factors affecting the regeneration and growth of bulblets in bulb-scale cultures of *Lilium rubellum* Baker. *Journal of the Japanese Society for Horticultural Science*, 1985, vol. 54, no. 1, p. 82-86.

PELKONEN, Veli-Pekka and KAUPPI, Anneli. The effect of light and auxins on the regeneration of Lily (*Lilium regale* Wil.) cells by somatic embryogenesis and organogenesis. *International Journal of Plant Sciences*, 1999, vol. 160, no. 3, p. 483-490.

RITALA, A.; MANNONEN, L. and OKSMAN-CALDENTEY, K.M. Factors affecting the regeneration capacity of isolated barley microspores (*Hordeum vulgare* L.). *Plant Cell Report*, July 2001, vol. 20, no. 5, p. 403-407.

ROBB, Sheila M. The Culture of Excised Tissue 3*Lilium speciosum* Thun. *Journal of Experimental Botany*, 1957, vol. 8, no. 3, p. 348-352.

SAVIDGE, R.A.; HEALD, J.K. and WAREING, P.F. Non-uniform distribution and seasonal variation of endogenous indol-3yl-acetic acid in the cambial region of *Pinus contorta* Dougl. *Planta*, June 1982, vol. 155, no. 1, p. 89-92.

SHARMA, Vijendra K.; HANSH, Robert; MENDEL, Ralf R. and SCHULZE, Jutta. Seasonal effect on tissue culture response and plant regeneration frequency from non-bombarded and bombarded immature scutella of barley (*Hordeum vulgare*) harvested from controlled environment. *Plant Cell, Tissue and Organ Culture*, April 2005, vol. 81, no. 1, p. 19-26.

TAKAYAMA, Shinsaku and MISAWA, Masanaru. Differentiation in *Lilium*, bulb scales grown *in vitro*. Effect of various cultural conditions. *Physiologia Plantarum*, June 1979, vol. 46, no. 2, p. 184-190.

TANIMOTO, Shizufumi and MATSUBARA, Yuuichiro. Stimulating effect of spermine on bulblet formation in bulb-scale segments of *Lilium longiflorum*. *Plant Cell Reports*, December 1995, vol. 15, no. 3-4, p. 297-300.

TIAN, Qing and REED, Jason W. Molecular links between light and auxin signaling pathways. *Journal of Plant Growth Regulation*, September 2001, vol. 20, no. 3, p. 274-280.

VARSHNEY, A.; DHAWAN, V. and SRIVASTAVA, P.S. A protocol for *in vitro* mass propagation of asiatic hybrids of lily through liquid stationary culture. *In vitro Cellular and Development Biology-Plant*, September 2000, vol. 36, no. 5, p. 383-391.

VARSHNEY, A.; LAKSHMIKUMARAN, M.; SRIVASTAVA, P.S. and DHAWAN, V. Establishment of genetic fidelity of *in vitro*-raised *Lilium* bulblets through RAPD markers. *In Vitro Cellular and Development Biology-Plant*, March 2001, vol. 37, no. 2, p. 227-231.

WAWROSCH, C; MALLA, P.R. and KOPP, B. Clonal propagation of *Lilium nepalense* D. Don, a threatened medicinal plant of Nepal. *Plant Cell Reports*, June 2001, vol. 20, no. 4, p. 285-288.

YAMAGISHI, Masumi. Effect of culture temperature on the enlargement, sugar uptake, starch accumulation, and respiration of *in vitro* bulblets of *Lilium japonicum* Thunb. *Scientia Horticulturae*, April 1998, vol. 73, no. 4, p. 239-247.