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Maturating, Enlarging and Breaking Dormancy of *In Vitro Lilium* Bulblets

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Abstract
One of the most effective ways to propagate *Lilium* is by using tissue culture techniques, preferably through bulblet production. In addition to the surviving percentage of *Lilium* bulbs after transplantation, the small size of *in vitro* bulblets compared to commercial bulbs and bulblets' dormancy are the most important constraints to commercializing *Lilium* micropropagation. Different concentrations of sucrose or sorbitol as carbohydrate sources were investigated as maturation and enlargement factors on *in vitro* bulblets of *Lilium longiflorum* cv. 'Ceb-dazzle'. Five concentrations of gibberellic acid and 4°C cold treatment for 6, 7, 8, 9, 10 and 11 weeks in the greenhouse were studied in relation to the dormancy breaking of three *in vitro Lilium* bulblets cultivars: 'Simplon', 'Navona' and 'Ceb-dazzle', which belong to the Oriental, Asiatic and LA hybrids. The results showed that 3% of sucrose and two weeks of culture significantly increased the bulblet maturation and enlargement of 'Ceb-dazzle'. One mg L^{-1} gibberellic acid for six weeks, or cold treatment for six to eight weeks, had significant positive effects on the dormancy breaking of *in vitro* bulblets of *Lilium* cultivars.

Key words: carbohydrate sources, cold treatment, commercial bulb, micropropagation

Abbreviations: **GA_3,** gibberellic acid; **NAA,** naphthaleneacetic acid; **BAP**, benzylaminopurine; **CRD,** completely randomized design; **LSD,** least significant differences

Introduction

Lilium is one of the most important ornamental plants worldwide, and is ranked within the top 10 flowers in the export market (Sharma *et al.*, 2005). Since 1979, the differentiation in *Lilium* bulb scales has been reported (Takayama and Misawa, 1979). *In vitro* propagation of some *Lilium* species and hybrids using either organogenesis or somatic embryogenesis has been carried out, e.g., *L. longiflorum* Thunb. (Bacchetta *et al.*, 2003; Nhut 2003; Nhut *et al.*, 2006; Liu *et al*., 2012), *L. speciosum* Thunb. (Chang *et al.*, 2000), *L. nepalensis* D.Don (Wawrosch *et al.*, 2001), *Lilium* oriental hybrids (Lian *et al.*, 2002; Han *et al.*, 2005), *Lilium* Asiatic hybrids (Varshney *et al.*, 2000; Lian *et al.*, 2003; Xiao-Hual *et al*., 2012), *L.× formolongi* K. Wada (Ho *et al.*, 2006), some *Lilium* species (Mori *et al.*, 2005), *L. ledebourii* (Baker) Boiss. (Memar moshrefi *et al.* 2002; Azadi and Khosh-Khui, 2007;

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Padasht Dehkaei *et al.*, 2008; Bakhshaie *et al.*, 2010) and *L. lancifolium* Thunb. (Sun *et al*., 2013). There are some restrictions to producing commercial bulbs through *in vitro* bulblets. Among them, the small size of *in vitro* bulblets (Han *et al.*, 2005) and dormancy are the most important barriers to be considered (Langens-Gerrits *et al.*, 2001; Dole, 2003). Temperature seems to be one of the factors in breaking dormancy (Delvallée *et al.*, 1990; Langens-Gerrits *et al.*, 2001). The dormancy of *in vitro* lily bulblets was broken by several weeks of low temperature (Langens-Gerrits *et al*., 2001, 2003). Furthermore, it was reported that endogenous levels of sucrose in bulbs are linked with dormancy development (Nowak *et al.*, 1974; Hobson and Davies, 1978; Aguettaz *et al.*, 1990; Xu *et al.*, 2006). Gibberellic acid (GA_3) has been shown to break dormancy in some bulbous plants, such as hyacinth (Tymoszuk *et al.*, 1979), garlic (Gua *et al.*, 2000; Rahman *et al.*, 2006) and *Lilium* (Ohkawa, 1979; Aguettaz *et al.*, 1990; Gerrits *et al.*, 1992). In this paper, different concentrations of carbohydrate sources, gibberellic acid and cold treatments were studied to determine the best treatments to produce commercial-sized *Lilium* bulbs.

Materials and Methods

Plant materials

Three *Lilium* L. cultivars, "Simplon", "Navona" and "Ceb-dazzle", belonging to Oriental, Asiatic and LA hybrids, respectively, were used for all the experiments.

Culture medium and conditions

In vitro bulblets were produced using central scales which were placed on MS basal medium supplemented with naphthaleneacetic acid (NAA) (0.3 mg L^{-1}), benzylaminopurine (BAP) (0.03 mg L^{-1}), Gamborge B5 vitamins and 7 g L^{-1} agar agar. Then, pH was adjusted to 5.8 before autoclaving at 121°C and 1.2 kg cm^{-1} pressure for 15 minutes. Then, the bulblets were placed in the 250 ml jam-jars containing 40 ml of medium for the maturation and enlargement experiments. The cultures were kept at 23±1ºC under a 16 h photoperiod at a light intensity of 45 µ mol m^{-2} s^{-1} supplied by cool-white fluorescent tubes. The *in vitro* bulblets were transferred to small disposable plastic pots (5 cm diameter) containing autoclaved peat-moss in the greenhouse at 24±2ºC. All pots were covered with transparent plastic bags for one week, which were gradually removed over the following week. All bulblets were acclimatized at the end of the second week.

Effects of different concentrations and types of carbohydrate on enlargement and maturation of in vitro bulblets of 'Ceb-dazzle'

In vitro bulblets of 'Ceb-dazzle' were cultured on Murashige and Skoog (MS) medium, supplemented with three concentrations of sucrose or sorbitol (1, 2 and 3%) as carbohydrate sources. Data were recorded after zero, two, four and six weeks of culture.

Effect of GA_3 on breaking dormancy of in vitro bulblets in three Lilium cultivars before and after acclimatization

In vitro bulblets of three *Lilium* cultivars ('Simplon', 'Navona' and 'Ceb-dazzle') were cultured on MS medium, supplemented with five different concentrations of GA_3 (0, 0.001, 0.01, 0.1 and 1 mg L^{-1}). Data were recorded at zero, two, four and six weeks of culture. The GA_3-treated *in vitro* bulblets were transferred to the greenhouse at 24±2°C for acclimatization. The number of green and yellow leaves, root number, number of scales per bulblet and bulblet diameter were recorded in the *in vitro* experiments. Leaf numbers and leaf length were recorded two weeks after acclimatization.

Effect of cold treatment on dormancy breaking of in vitro bulblets in three Lilium cultivars

In vitro bulblets of the above cultivars were exposed to 4°C cold treatment for six, seven, eight, nine, 10 and 11 weeks, then transferred to the greenhouse at 24±2°C. Leaf numbers and means of leaf length were recorded two weeks after acclimatization.

Flowering evaluation of in vitro bulblets in three Lilium cultivars

Flowering percentage was measured 16 months after acclimatization.

Statistical analysis

Factorial experiments based on a completely randomized design (CRD) with five replications were used for data analysis. The means were compared using LSD Tests at 5% level.

Results

Effects of different concentrations and type of carbohydrates on enlargement and maturation of in vitro bulblets of 'Ceb-dazzle'

The largest and the highest number of scales per bulblet were achieved with 3% concentration of carbohydrate, while the number of green leaves decreased (Table 1). No significant difference was observed with different concentrations of sorbitol and sucrose on enlargement and maturation of *in vitro* bulblets' parameters (Table 1). After two weeks of culture, significant growth of *in vitro* bulblets was observed, while keeping the bulblets for a longer period of time did not increase the size of the bulblets (data not shown).

Table 1. Effects of type and concentration of carbohydrates on maturation and enlargement of *in vitro* bulblets of *Lilium longiflorum* 'Ceb-dazzle'.

Type of carbohydrate	Carbohydrate concentration (%)	Number of root per bulblet	Number of green leaves	Number of yellow leaves	Bulblets diameter (mm)	Number of scales per bulblet
sorbitol	1	4.10±0.29†	3.52±0.28	1.06±0.17	4.04±0.24	2.44±0.28
	2	4.53±0.33	3.76±0.27	2.06±0.27	4.26±0.26	1.76±0.12
	3	3.71±0.21	2.45±0.24	1.10±0.15	4.48±0.21	2.50±0.18
sucrose	1	3.75±0.29	3.55±0.26	0.93±0.14	3.60±0.13	1.96±0.13
	2	4.25±0.18	3.11±0.15	0.90±0.16	3.87±0.20	2.22±0.15
	3	4.58±0.29	2.78±0.20	1.51±0.19	5.03±0.21	2.58±0.22

†Values represent the mean ± SE

Effect of GA_3 on dormancy breaking of in vitro bulblets in three Lilium cultivars before and after acclimatization

The results showed that 1 mg L^{-1} GA_3 produced the largest bulblet diameter and the highest number of roots and green leaves in all cultivars (Table 2). After six weeks of culture, the same result was obtained for the same concentration of GA_3 (Table 3).

Increasing the gibberellic acid concentration enhanced leaf numbers in all three cultivars significantly after transferring to greenhouse.

Table 2. Effects of gibberellic acid (GA3) concentrations on maturation, enlargement and dormancy breaking of *in vitro* bulblets of three *Lilium* cultivars before and after acclimatization.

Cultivar	GA3 concentration (mg L^{-1})	Number of root per bulblet	Number of Green leaves before acclimatization	Number of Yellow leaves before acclimatization	Number of total leaves after acclimatization	Leaf length after acclimatization (cm)	Bulblets diameter (mm)	Number of scales per bulblet
'Ceb-dazzle'	0.00	4.11±0.53†	3.58±0.46	1.30±0.16	1.58±0.14	57.69±11.31	4.55±0.58	3.50±0.45
	0.001	4.61±0.59	4.18±0.53	1.33±0.17	1.84±0.28	59.56±10.53	4.78±0.61	3.66±0.47
	0.01	4.70±0.60	4.53±0.58	1.56±0.20	1.66±0.22	63.35±12.42	4.41±0.56	3.06±0.39
	0.10	4.25±0.57	4.33±0.58	1.50±0.20	1.53±0.14	58.80±11.11	4.09±0.55	2.31±0.31
	1.00	4.91±0.63	5.53±0.71	2.28±0.29	1.86±0.15	52.73±7.86	4.95±0.63	2.66±0.34
'Navona'	0.00	3.72±0.54	3.61±0.52	0.63±0.09	1.90±0.23	49.44±9.34	4.21±0.61	2.83±0.41
	0.001	4.83±0.74	4.23±0.65	1.19±0.18	2.77±0.49	51.51±8.96	5.16±0.79	3.26±0.50
	0.01	3.91±0.57	4.68±0.68	1.17±0.17	3.10±0.17	48.66±7.60	4.87±0.71	3.27±0.47
	0.10	3.53±0.45	4.71±0.60	1.23±0.15	3.00±0.28	49.45±6.18	4.78±0.61	3.10±0.40
	1.00	4.63±0.59	4.65±0.60	1.35±0.17	3.25±0.29	47.18±5.72	5.18±0.66	2.65±0.34
'Simplon'	0.00	4.17±0.58	3.33±0.46	1.92±0.26	1.52±0.13	50.73±6.86	4.58±0.64	2.13±0.29
	0.001	4.88±0.66	3.27±0.44	1.27±0.17	2.75±0.45	48.44±7.24	5.74±0.78	2.61±0.35
	0.01	3.55±0.48	2.31±0.31	1.05±0.14	2.80±0.42	52.51±7.85	5.44±0.74	2.64±0.35
	0.10	4.73±0.61	2.91±0.37	0.98±0.12	2.84±0.25	56.17±6.62	5.50±0.71	2.95±0.38
	1.00	6.77±0.92	5.01±0.68	2.10±0.28	3.00±0.30	59.27±9.25	5.88±0.80	2.83±0.38

†Values represent the mean ± SE.

Table 3. Effect of culture period (in weeks) on maturation, enlargement and dormancy breaking of *in vitro* bulblets of three *Lilium* cultivars.

Cultivar	Weeks after culture	Number of root per bulblet	Number of Green leaves	Number of Yellow leaves	Bulblets diameter (mm)	Number of scales per bulblet
'Ceb-dazzle'	0	2.88± 0.33†	3.54±0.4	0.24±0.20	3.69±0.42	3.65±0.42
	2	4.26±0.49	4.25±0.49	1.86±0.21	4.48±0.51	3.02±0.34
	4	5.25±0.61	4.81±0.56	2.05±0.24	4.91±0.57	2.66±0.31
	6	5.80±0.68	5.16±0.60	2.27±0.26	5.22±0.61	2.86±0.33
'Navona'	0	3.30±0.39	3.74±0.44	0.00	4.35±0.51	3.48±0.41
	2	4.04±0.48	4.20±0.50	1.13±0.13	4.79±0.58	3.05±0.36
	4	4.49±0.58	4.59±0.59	1.57±0.20	5.06±0.65	2.77±0.36
	6	4.76±0.61	5.27±0.68	2.03±0.26	5.28±0.68	2.60±0.33
'Simplon'	0	4.02±0.46	2.54±0.29	0.00	4.66±0.53	3.30±0.38
	2	4.65±0.54	3.22±0.37	1.63±0.19	5.33±0.62	2.55±0.30
	4	5.14±0.64	3.63±0.45	2.06±0.25	5.88±0.74	2.33±0.29
	6	5.68±0.71	4.22±0.53	2.41±0.30	6.04±0.76	2.28±0.28

†Values represent the mean ± SE.

Effect of cold treatment on dormancy breaking of in vitro bulblets in three Lilium cultivars

Imposition of more than six weeks of cold treatment produced longer leaves with a simultaneous reduction in the number of leaves (Table 4).

Flowering evaluation of in vitro bulblets in three Lilium cultivars

All cultivars flowered 16 months after acclimatization (Fig. 1). The flowering percentages were 2, 23 and 19 in 'Ceb-dazzle', 'Navona' and 'Simplon', respectively. No significant differences were observed between 1 mg L^{-1} of GA_3 and six to eight weeks of cold treatment (data not shown).

Table 4. Effect of cold treatment on dormancy breaking of *in vitro* bulblets of three *Lilium* cultivars.

Cultivar	Number of cold weeks	Leaf number	Leaf length (cm)
'Ceb-dazzle'	6	1.75±0.50†	51.63±14.9
	7	1.25±0.27	35.31±7.89
	8	2.09±0.63	41.34±12.46
	9	1.32±0.26	50.81±10.16
	10	1.75±0.50	67.56±19.50
	11	1.28±0.34	90.86±24.28
'Navona'	6	3.14±0.68	44.53±9.71
	7	3.78±0.71	45.11±8.52
	8	3.63±0.83	45.43±10.42
	9	2.40±0.53	64.02±15.19
	10	2.32±0.43	80.39±9.51
	11	2.59±0.45	53.82±11.31
'Simplon'	6	2.51±0.45	63.01±13.19
	7	1.91±0.31	79.16±7.15
	8	1.56±0.23	47.47±17.76
	9	1.88±0.36	90.58±28.06
	10	1.53±0.39	108.71±30.25
	11	1.27±0.29	128.38±5.89

†Values represent the mean ± SE

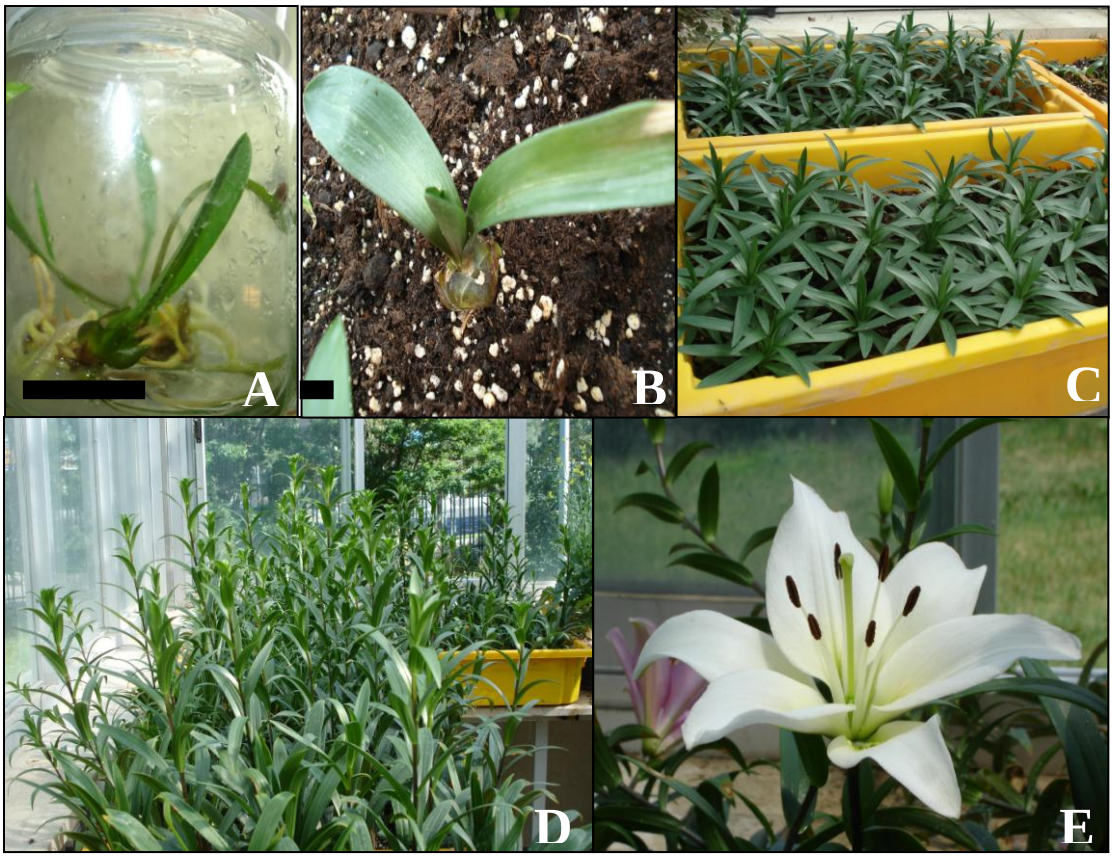

Fig. 1. 'Simplon' cultivar: *in vitro* bulblet A) 10 months after acclimatization, B) one year after acclimatization, C) 14 months after acclimatization, D) flower of 'Simplon' 16 months after acclimatization. Bar = 1 cm.

Discussion

The small size of *in vitro* bulblets, the low percentage of survival during acclimatization and *in vitro* bulblets' dormancy are the most important barriers for establishment of a micropropagation protocol for *Lilium* (Langens-Gerrits *et al*. 2001; Dole 2003; Bong *et al*. 2005). Therefore, studies on maturation and enlargement of *in vitro* bulblets and on breaking dormancy are necessary in order to produce commercial-sized *Lilium* bulbs.

The positive effect of carbohydrates on *in vitro* bulblet enlargement and maturation was confirmed in this research. Sucrose is the commonest carbohydrate in the phloem sap of plants and the general carbohydrate for *in vitro* propagation of many plants (Murashige and Skoog 1962; Lemos and Baker, 1998; Fuentes *et al.*, 2000). There was no significant difference between sucrose and sorbitol in our experiments for the growth parameters. Due to availability and the lower cost of sucrose compared to sorbitol, sucrose has been chosen as a carbon source for maturation and enlargement of lily bulblets.

The effect of sucrose concentration on three Oriental *in vitro Lilium* bulblets and *L. longiflorum* showed that a high concentration of sucrose (9%) was the best treatment for the growth of bulblets (Liu *et al.*, 2008). Similar results were achieved by Kumar *et al*. (2005). Our findings showed that the concentration of carbohydrates is the main factor affecting bulblet growth, rather than the type of carbohydrate sources. Among the different concentrations of carbohydrates, 3% carbohydrates for two weeks were adequate for *in vitro* bulblet enlargement. Despite the fact that higher concentrations

of sucrose as a carbohydrate source in the media showed positive effects on the *in vitro* bulblets' enlargement and maturation, negative effects of increasing the carbohydrate concentration have been reported on the *in vitro* bulblet production. A higher concentration of sucrose increased cormel induction and number of cormels in *Gladiolus*, as well as bulblet induction in several Korean native lilies (Dantu and Bhojwani, 1995; Jeong, 1996).

Gibberellic acid is one of the important plant growth regulators that can improve seed germination percentage and plant growth rate (Finkelstein *et al.*, 2008). It has also been known to break dormancy in different organs of many plants. Gibberellic acid broke dormancy in *hyacinthus orientalis* L. and *Sedum bulbiferum* bulbs (Tymoszuk *et al.*, 1979; Terui and Okagami, 1988). In the present study, the positive effect of rising GA_3 concentration on breaking dormancy of *in vitro Lilium* bulblets and stimulation of bulblets' growth after acclimatization was also confirmed, in agreement with previous studies on *L. speciosum* Thunb. (Ohkawa, 1979; Aguettaz *et al.*, 1990; Gerrits *et al.*, 1992).

Considering the dormancy level of bulblets, a long period of culture at a low temperature was necessary to induce 100% sprouting of *Lilium* bulblets (Delvallée *et al*., 1990; De Klerk *et al*., 1992; Langens-Gerrits *et al*., 2001). During this phase, the tissue is prepared not only for sprouting but also for bulb growth. Langens-Gerrits *et al*. (2003) reported that the longer the period of cold storage, the faster and the more uniform the leaf emergence occurring in *in vitro* bulblets of different lily genotypes. However, our results showed a decrease in the number of leaves that emerged when bulblets were subjected to more than eight weeks of cold treatment. It was also suggested that the exposure of *in vitro* developed bulblets of *L. rubellum* Baker to low temperature is necessary to initiate the flowering process (Ishimori *et al*., 2009). It can be concluded that the best cold storage (4°C) treatment for these cultivars was six to eight weeks. At six weeks of culture, the medium supplemented with 1 mg L^{-1} gibberellic acid was as good as six to eight weeks of cold treatment for breaking dormancy.

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