Effects of Cold Treatments on Seedling Emergence and Growth of *Lilium davidii* var. *unicolor* Bulblets

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Abstract. For *Lilium davidii* var. *unicolor* bulblets produced by scale propagation, the effects of cold treatments on the sprouting and development of bulblets were studied. The results showed that 5 °C was a more suitable temperature than 2 or 10 °C. Bulblets treated at 5 °C for 3 weeks presented the best uniformity of seedling emergence, and the sprouting rate was 100%. Moreover, the largest bulbs were observed in this treatment after a growing season. It was found that long storage at low temperatures is unfavorable for bulb development. The weight and circumference of bulbs from bulblets that were cold-treated for more than 5 weeks were significantly less than those treated for 1 to 4 weeks. During the first 4 weeks of cold storage, the starch content of bulblets decreased significantly, coinciding with an increase in soluble sugars. The starch and soluble sugar contents in bulblets stored at 2 and 5 °C changed faster than those in bulblets stored at 10 °C. However, the effect of temperature on carbohydrates diminished gradually as the storage time increased. Long storage of bulblets at low temperatures is not good for subsequent growth and development. The results of this study provide important information for accelerating the scale propagation of *L. davidii* var. *unicolor* and maximizing bulb yield.

Lilium davidii var. *unicolor*, a variety of *Lilium davidii* Elwes, is an important bulbous plant not only for its beautiful flowers, but also for its edible bulbs. It has a long history of being used as a vegetable in China. The bulbs taste sweet and can be eaten fresh or cooked. Bulbs are a healthy food that helps strengthen people's immune system because the bulbs are rich in starch, dietary fiber, vitamins, and a variety of bioactive substances, such as polysaccharides, alkaloids, and saponins (You et al., 2010; Zhang et al., 2010). Moreover, bulbs have been suggested to be useful in relieving pulmonary-related diseases such as cough and phlegm, and could be used as a therapeutic candidate for pulmonary inflammation caused by cigarette smoke (Lee et al., 2013; Li et al., 2021).

Among the reproduction methods of lilies, scale propagation is preferred because it is the

most cost-effective and time-saving approach in the large-scale production of bulbs (Matsuo and van Tuyl, 1984; Matsuo et al., 1989; Tang et al., 2020). A large number of bulblets can be obtained through scale propagation. In research on bulblet differentiation after scale propagation of *L. longiflorum*, it was found that 1.2 to 2.6 bulblets per scale were obtained from external to internal scales (Marinangeli et al., 2003). Our previous research showed that scales of *L. davidii* cultivated at 30 °C could produce approximately two bulblets per scale, and the propagation coefficient per bulb could reach 111.9 (Jia et al., 2020). After the production of bulblets, successful sprouting and growth becomes the key issue for further bulb production. The developmental state of the bulblet, bulblet size, and degree of dormancy were considered three factors with a major influence on the growth of the bulblets (Langens-Gerrits et al., 2003). Bulbs of lilies develop dormancy to survive unfavorable environments. Bulbs can sprout and grow under suitable environmental conditions after dormancy is broken (Xu et al., 2006). Temperature, carbohydrates, and hormones are the main factors involved in the development of dormancy (Delvallée et al., 1990). Many studies have reported the conversion of carbohydrates, and variations in hormones and related enzymes during dormancy development (Djilianov et al., 1994; Liu et al., 2014; Xu et al., 2006).

Because the growth cycle of the lily requires a cold–warm–cold temperature change (Zhou

et al., 2021), bulblets generated on scales may develop dormancy depending on the culture conditions (Delvallée et al., 1990). Cold treatment can break the dormancy of lily bulblets, which leads to sprouting and plant development (Shin et al., 2002). Normally, 5 °C can break dormancy effectively for many geophytes, including lily (Dole, 2003). However, the response of plants to low temperatures varies greatly as a result of differences in species, cultivar, plant maturity, cultivation method, and environmental conditions (Erwin and Engelen-Egles, 1998; Kawarabayashi, 2015; Lee et al., 1996). Langens-Gerrits et al. (2003) studied the effect of low temperature on sprouting, time of leaf emergence, and bulb growth of lily bulblets generated in vitro. Micropropagated bulblets of *L. speciosum* planted at 15 °C showed a greater sprouting percentage than those planted at 20 and 25 °C (de Klerk and Paffen, 1995).

When bulblets that are generated on scales are planted in soil, the major problems are low emergence rate and seedling uniformity. In our study, we investigated the effect of different temperatures and treatment durations on the sprouting of bulblets, and bulb growth was assessed after a growing season. This study aimed to screen the best temperature treatment for bulblets of *L. davidii* produced by scale propagation that resulted in maximum sprouting under a minimum storage time and that produced the largest bulbs. The results of this study will provide theoretical and technical support for bulb production of *L. davidii* var. *unicolor* by scale propagation.

Materials and Methods

Plant materials. Bulbs of *L. davidii* var. *unicolor* were used for producing bulblets by means of scale propagation. Fresh bulbs (150 ± 10 g) were collected in Mar. 2019 from a field at the Plateau Flower Research Center of Qinghai University, Qinghai Province, China (lat. 36°43'32"N, long. 101°45'2"E; elevation, 2320 m). Bulbs were placed at room temperature for 1 week before they were used for scale propagation. Scales were removed from healthy bulbs, washed in tap water, and immersed in 0.5% thiophanate-methyl for 30 minutes. Scales that had mechanical damage were removed. Peat (Floragard, Niedersachsen, Germany) was sterilized with 125× formaldehyde and 50% thiophanate-methyl (100 g/m³). Then, scales were mixed with an equal volume of the prepared peat, placed in a propagation bed, and covered with 5 cm of sterilized peat. To preserve moisture, the bed was wrapped in a plastic film with holes. Scales were incubated at 30 °C. The temperature of the propagation bed was maintained using a heater wire and temperature controller. During incubation, bulblet growth was checked every week. After 6 weeks, bulblets were collected and graded according to their weight and size. Bulblets with a uniform size (circumference, 1.0 ± 0.1 cm; weight, 0.2 ± 0.02 g) were selected for further experiments.

Cold treatment of the bulblets. Effects of different cold treatments (2, 5, and 10 °C) on seedling sprouting and bulb growth of *L. davidii* var. *unicolor* were investigated in this

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study. Bulblets without cold treatment (room temperature, $20 \pm 2^\circ\text{C}$) were used as a control. Bulblets were stored at different temperatures for 8 weeks. Thirty bulblets were removed and planted in a crate ($60 \times 40 \times 18$ cm) every week. Three replicates were performed for each treatment. Plastic film with holes (10×40 mm) was laid in the bottom of crates, which were then filled with peat. The bulblets were planted, covered with 3 cm peat, and then placed outdoors. The temperature was 9 to 18°C , the relative humidity of the air was 45% to 70%, the illumination intensity was 3×10^4 to 4×10^4 lux, and shading was used at noon. The moisture of the peat was kept at $\approx 50\%$ to 60% during the growing process.

Carbohydrate metabolism. Every week, 30 bulblets were selected randomly from each treatment and pooled to determine the contents of soluble sugars and starch. The contents of starch and soluble sugars were determined using anthrone colorimetry and were estimated based on fresh weight (Jan and Roel, 1993). Three independent experiments were performed.

Data collection and statistical analyses. Sprouting of bulblets was recorded every day, and the rate of emergence was calculated. After a growing season, the weight and circumference of bulbs were measured for each crate. Analysis of variance was performed using PASW statistics 18, and significant differences between treatments were assessed using the least significant difference test at $P < 0.05$.

Results

Effects of cold treatments on the time of sprouting. The different temperature treatments showed various influences on the days to final sprouting, but the initial emergence time was unaffected (Table 1). Bulblets without cold treatment showed slow and nonuniform sprouting that lasted for more than 56 d. For bulblets stored at low temperatures, the days from planting to final sprouting were greatly shortened. Wide variation was observed for days to final sprouting among different cold treatments. Bulblets that were treated at 5°C for 3 weeks finished their sprouting in 21 d. The sprouting phase took 6 weeks to complete. Bulblets stored at 2°C for 4 weeks finished sprouting in 20 d. The sprouting phase took 7 weeks to complete. However, for bulblets treated at 10°C , it took 29 d to finish sprouting, even if they were pretreated for 5 weeks. The total time spent sprouting was much longer for the 10°C treatment than that for the 5°C and 2°C treatments. In conclusion, considering the time and uniformity of seedling emergence, the best temperature and storage time for bulblet pretreatment is 5°C and 3 weeks.

Effects of cold treatments on the sprouting rate. The sprouting rates for different temperature treatments are shown in Figs. 1 through 3. In general, cold treatment significantly enhanced the sprouting percentage of *L. davidii* bulblets generated by scale propagation. Different temperatures and storage times

Table 1. Effects of low temperature and storage time on seedling sprouting.

Duration of cold treatment (wk)	Sprouting time at 2°C (d)		Sprouting time at 5°C (d)		Sprouting time at 10°C (d)	
	First sprouting	Final sprouting	First sprouting	Final sprouting	First sprouting	Final sprouting
0 ^z	6	>56	6	>56	6	>56
1	5	>56	5	36	5	>56
2	8	42	4	35	5	50
3	6	35	6	21	6	35
4	6	20	6	22	7	34
5	7	21	7	20	5	29
6	7	21	5	20	5	27
7	6	21	4	22	6	28
8	6	23	6	21	5	28

^zBulblets without cold treatment.

showed different influences. For bulblets without cold treatment, maximum sprouting (78.89%) occurred at 35 d after cultivation. Under a constant 2°C treatment, the bulblets presented different sprouting performance when the storage time was extended (Fig. 1). The maximum sprouting rate of bulblets stored for 1 week was 93.33%, which occurred 35 d after planting. The percentage of emergence increased to 100% when the storage time of bulblets was more than 2 weeks. To reach the greatest sprouting rate (100%), bulblets stored at 2°C for 2 weeks and 3 weeks needed 49 d and 42 d to sprout, respectively. Four weeks was the optimum storage time for treatment at 2°C , because it took only 28 d for the emergence of all bulblets. However, sprouting uniformity did not improve with the extension of storage time. Bulblets needed 21 to 28 d to finish sprouting if the storage time was longer than 5 weeks, which indicates that the total time taken for seedling emergence was longer.

Bulblets treated at 5°C showed similar variation as those treated at 2°C (Fig. 2). On the seventh day after the bulblets were planted in soil, the sprouting percentages were 1.11% to 15.56% for different storage times. The emergence of seedlings increased dramatically after 7 d of planting. The greatest sprouting

rate (100%) was observed at 42 d for bulblets stored at 5°C for 1 week and 2 weeks. However, only 21 d was needed for bulblets in the 3-week treatment to reach the 100% sprouting percentage. Thus, 3 weeks was the best storage time for bulblets stored at 5°C when considering emergence performance.

At a constant 10°C , the greatest sprouting rates of bulblets stored for 1 week and 2 weeks were 90% and 97.78%, respectively. Compared with untreated bulblets, those stored in low-temperature treatment showed enhanced seedling emergence (Fig. 3). However, compared with bulblets at 2 and 5°C , bulblets at 10°C needed more time to sprout fully. For the $10^\circ\text{C}/3\text{-week}$ and $10^\circ\text{C}/4\text{-week}$ treatments, the maximum sprouting rate (100%) occurred at 42 d and 35 d after planting, respectively.

Growth of bulblets after a growing season. Weight and circumference are important parameters in the evaluation of bulb growth. In our study, under the influence of low temperatures in the time range of 0 to 8 weeks, the variation in bulb weight and circumference was determined after a growing season for all treatments. Our results show that the storage time of the low-temperature treatment had a noticeable influence on bulb growth.

Fig. 1. Effects of 2°C treatment on the sprouting rate of bulblets. Values represent the mean of three replicates. Bars indicate SE. CK, bulblets without cold treatment; W, week.

Fig. 2. Effects of 5°C treatment on the sprouting rate of bulblets. Values represent the mean of three replicates. Bars indicate SE. CK, bulblets without cold treatment; W, week.

Low-temperature treatment over a certain time was not good for bulb growth. The storage time at the 2°C treatment had a significant influence on bulb weight. The postgrowing season bulb weight for bulblets stored at 2°C for 3 weeks and 4 weeks (1.84 g/bulb and 1.68 g/bulb) was significantly heavier than that of control bulbs and those treated for longer than 5 weeks. Moreover, storage time also had a dramatic effect on both the weight and circumference of bulblets treated at 5°C (Figs. 4 and 5). After a growing season, the bulb weight and circumference of bulblets stored longer than 5 weeks were significantly less than those of bulblets pretreated for 1 to 4 weeks. Maximum bulb weight and circumference were observed for the 5°C/3-week (4.2633 cm, 2.0533 g) and 5°C/4-week (4.1233 cm, 2.3467 g) treatments. This result suggests that the storage time should

not exceed 4 weeks when performing cold treatments on *L. davidii* bulblets. Similarly, bulblets pretreated for more than 5 weeks at 10°C also showed weaker growth than the control bulblets and bulblets treated for 1 to 4 weeks. The largest bulbs were also observed at 3 and 4 weeks.

Carbohydrate metabolism. The enhancing effect of cold treatment on seedling emergence and subsequent bulb growth may be a result of the transfer of sugars used in sprouting and photosynthetic processes after planting. During storage at low temperatures, starch breakdown coincided with an increase in soluble sugars (Fig. 6). Different temperature treatments showed dramatic effects on the starch content in bulblets. The variation in starch in bulblets stored at 2 and 5°C was similar and decreased quickly during the first

4 weeks. This value was significantly less than that for bulblets at 10°C after pretreatment for 3 weeks and 4 weeks. The variation in the starch content in bulblets at 2, 5, and 10°C was not significantly different from 5 weeks to 8 weeks. This result indicates that the effect of temperature on the starch content in bulblets diminished gradually over time. Moreover, it was found that the starch content in untreated bulblets broke down slowly and was greater than that in low-temperature-treated bulblets.

Soluble sugars accumulate as starch breaks down. Soluble sugars provide energy during bulb growth and development. At the same storage time, different temperatures had significantly different effects on the content of soluble sugars. In general, the content of soluble sugars in bulblets stored for 1 to 4 weeks at low temperatures increased rapidly, and the maximum content was observed at 4 weeks. When stored from 2 to 8 weeks, the soluble sugar content of bulblets at 2 and 5°C was significantly greater than that of the control and the 10°C-treated bulblets. The lower the temperature, the faster the conversion of soluble sugars, and the greater the soluble sugar content. It was found that at the eighth week of cold storage, the soluble sugar content in bulblets declined, which may be a result of the bulblets starting to consume soluble sugars. Therefore, for bulblets generated by scale propagation, long storage under low temperature is unfavorable for subsequent growth and development.

Discussion

Vegetative propagation by scaling is the most cost-efficient and time-saving approach to increase clones of lilies. In the bulb production of *Lilium davidii* var. *unicolor*, this technique is widely used among commercial growers because of its high propagation coefficient. Scales are broken off from the basal plate of clean bulbs to permit the formation of bulblets. Direct planting of scales in the field is not recommended because ≈17 weeks are required for bulblet development, during which extreme weather will definitely affect bulblet yield (Austin-McRae, 1998). Moreover, soil moisture is a critical condition for bulblet formation and development, but this is difficult to control in outdoor planting. The incubation method adopted in our study could reduce the propagation cycle to 6 weeks, and the propagation coefficient largely increased. In our previous study, we screened the optimum temperature for scale propagation of *L. davidii* var. *unicolor* and investigated the effects of plant growth regulators on bulblet development (Tang et al., 2020). After succeeding in obtaining a large number of bulblets, improving the seedling sprouting rate is the key to increasing subsequent bulb production. Low sprouting rates and seedling uniformity were observed when bulblets were planted in the field immediately after incubation. This is because bulblets are generated under relatively high temperatures (25 to 30°C), and they may enter dormancy in

Fig. 3. Effects of 10°C treatment on the sprouting rate of bulblets. Values represent the mean of three replicates. Bars indicate SE. CK, bulblets without cold treatment; W, week.

Fig. 4. Effects of low-temperature treatments on the circumference of bulbs. Values represent the mean of three replicates. Bars indicate SE. W, week. The different lowercase letters indicate significant differences at $P < 0.05$ level.

response to unsuitable environmental conditions (Delvallée et al., 1990). Dormancy release is one of the most important events in the production of lilies. Although basic patterns for breaking dormancy have been proposed, the procedures are not exactly the same in different species (Dole, 2003). Dormancy can be broken by low temperatures, plant growth regulators, and other treatments, such as nitric oxide and low oxygen (Amaki and Amaki, 2005; Jásik and de Klerk, 2006; Niimi et al., 1988; Niu et al., 2015; Situma et al., 2015). Low temperature is the most common way to break dormancy for geophytes. The period of storage at low temperature affects not only the number of sprouting bulblets, but also the time it takes for the seedling to emerge after planting in the field. The results of our study show that low temperature has a significant effect on enhancing the sprouting percentage of *L. davidii* bulblets. A constant

5°C treatment for 3 weeks is the best selection for bulblet pretreatment before planting, at which only 21 d are needed for sprouting of all bulblets. As an alternative, storage at 2°C for 4 weeks is also effective in promoting seedling emergence. Cold treatment longer than 5 weeks is not helpful for increasing the sprouting of bulblets. Instead, the bulb weight and circumference are dramatically less than those stored for up to 4 weeks.

The dormancy of many plants can be broken by low-temperature treatments. For *L. davidii*, Sun et al. (2004) found that after 101 d of storage at 2°C, all bulbs sprouted within 20 d. This presents a difference between mature bulbs and bulblets. In our study, bulblets only needed 4 weeks of 2°C treatment to achieve 100% sprouting. Previous studies have been performed on the dormancy release of bulblets regenerated on scales in vitro, but no study has been reported for bulblets

produced by scale propagation. de Klerk (2009) found that 9°C was the optimal temperature for breaking dormancy in lily bulblets obtained by microporopagation. Storage for several weeks at 5°C promoted dormancy release in bulblets that regenerated from scale explants of *L. speciosum* (Langens-Gerrits et al., 2003). For Asian lilies, storage at a temperature ranging from 1 to 10°C was effective for breaking the dormancy of bulblets regenerated in vitro (Shin et al., 2002). However, shoot emergence was not influenced by temperature treatments given to bulbils of *Lilium × elegans* Thunb. (Suh and Roh, 2014). The genus *Lilium* has abundant species/varieties, and it can be seen that the temperature and storage time required for dormancy release vary across species, varieties, organs, and stages of growth.

The bulb is the vegetative storage organ of lilies, in which starch is the major reserve carbohydrate. Exposure of bulbs to low temperatures induces starch hydrolysis accompanied by the accumulation of soluble sugars, and energy is provided for bulb dormancy release (Miller and Langhans, 1990; Xu et al., 2006). The regulatory pathways of carbohydrate metabolism are activated, and carbohydrates present a positive relationship with dormancy release in lily bulbs (Wang et al., 2018; Zhang et al., 2011). In our study, during the storage of bulblets at low temperatures, starch breakdown coincided with an increase in soluble sugars (Fig. 6). Storage at 2 and 5°C resulted in more dramatic alterations in bulb carbohydrates than storage at 10°C. Large changes in carbohydrates occurred during the first 4 weeks of storage. The effect of low temperatures on carbohydrates in bulblets diminished gradually over time. Long storage under low temperatures is unfavorable for the subsequent growth of seedlings because the bulbs start consuming carbohydrates during the later stage of storage. During dormancy breaking, similar carbohydrate changes were observed in bulbs of *L. pumilum* (Liu et al., 2016) and tulip (Kamenetsky et al., 2003), in tubers of potato (Claassens, 2002), and in flower buds and shoots of Japanese pear (Ito et al., 2012). The changes in carbohydrates during dormancy release may affect not only the leaf emergence of bulblets, but also the subsequent growth of bulbs. Starch hydrolysis and soluble sugar accumulation occurred mainly in the 0- to 4-week cold treatments, and the changes were no longer significant after 4 weeks. Excellent sprouting of bulblets was observed in cold treatments with a storage time of 3 to 4 weeks. Moreover, after a growing season, the largest bulbs were also obtained from bulblets that were stored for 3 weeks and 4 weeks at different low temperatures. Reserves of a certain amount of carbohydrates are important in the development of organs such as bulbs and tubers (Burton et al., 1992; Fernie and Willmitzer, 2001; Geigenberger, 2003). It was observed in *Tulipa edulis* that soluble sugar levels declined whereas starch and protein accumulated in the process of stolon development into a new bulb (Miao et al., 2016).

Fig. 5. Effects of low-temperature treatments on the weight of bulbs. Values represent the mean of three replicates. Bars indicate SE. W, week. The different lowercase letters indicate significant differences at $P < 0.05$ level.

Fig. 6. Variation in starch and soluble sugar (sol.) concentrations in bulbets during storage at low temperatures (2, 5, and 10°C). Values represent the mean of three replicates. CK, bulbets without cold treatment; W, week.

During tuber development in potato and sweetpotato, large amounts of carbohydrate reserves, such as starch and storage proteins, accumulate in the tubers (Firon et al., 2013; Kloosterman et al., 2008). Thus, excessively long storage is not good for bulb growth, possibly because of the overconsumption of carbohydrates in the bulbets.

In conclusion, to obtain the largest sprouting rate in the shortest time period and produce the largest bulbs after a growing season, the best temperature treatment for bulbets of *L. davidii* var. *unicolor* generated by scale propagation is 5°C for 3 to 4 weeks. The results of our study provide important information for accelerating the scale propagation of *L. davidii* var. *unicolor* and maximizing bulb yield.

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