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两步外植体法高频再生麝香百合

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摘要：采用两步外植体法，即以百合鳞片叶为初始外植体，以从初始外植体上长出的芽为次级外植体，成功建立了麝香百合的高频离体再生系统。对不同的BA浓度及次级外植体的不同部位对再生效果的影响，以及组培苗移栽前低温处理的影响进行了研究。结果表明：不同部位的次级外植体中，以短缩茎切片出芽快、整齐、芽数多且粗壮；以MS附加1.0 mg L^{-1} BA和0.1 mg L^{-1} NAA的培养基最适于麝香百合的分化。一个中等大小已脱春化的鳞茎通过两步外植体法能扩繁出54 000株左右的新植株，从鳞片叶开始至开花仅需8个月，而且4℃低温处理对开花期的影响不大。

关键词：麝香百合；组织培养；成芽；开花
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A Two-step Method for the Efficient Micropropagation of *Lilium longiflorum*

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Abstract: Bulb scale segments of *Lilium longiflorum* were used as original explants and shoot sections excised from shoot clusters arosed from the first explants as the secondary explants. The effects of different concentrations of BA (benzyladenine) and different parts of plant segments on shoot formation were studied. After roots formed, plantlets were treated at 4℃ for 0, 7 and 30 days. The results indicated that MS medium supplemented with 1.0 mg L^{-1} BA and 0.1 mg L^{-1} NAA was optimum for shoot formation and proliferation by using short basal stem segment as the second explant. Moreover, cold treatment had little effect on flowering stage. About 54 000 new plantlets could be obtained from one bulb. It took only 8 months from bulb scale to flowering.

Key words: *Lilium longiflorum*; Tissue culture; Shoot formation; Flowering

Lilium species (Liliaceae) are one of the three major flower bulbs in the commercial market[1] and chosen as fresh cut flowers for their attractive colours. Lilies are usually propagated by scaling, a technique which produces 3-5 bulbs from each bulb scale, depending on species, cultivar, and scale size. Scale propagation produces limited numbers of bulbs[2]. Therefore, micropropagation *in vitro* is essential to produce large numbers of bulblets in lily within a short period of time.

In the micropropagation of *Lilium* species, there were many studies on the regeneration of lily by using bulb scale fragments[2-6], leaves[7], anthers[8], flower filaments[9-10], stems and tepals[11] to initiate cultures.

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However, bulb scales *in vitro* were used as the best explant source for the production of bulblets [3-5, 12-15]. Han et al. [6] documented a new method by the formation of shoots with abnormally swollen basal plates and the regeneration system had been greatly improved. However, the exact number of new plantlets obtained by micropropagation techniques remains unknown. Moreover time and labor cost are the main limiting factors in micropropagation.

In order to improve the micropropagation efficiency of *Lilium longiflorum*, we had developed a two-step explant method, i.e. bulb scale segments were used as original explants and shoot sections excised from shoot clusters arosed from the first explants were used as the secondary explants to micropropagate new plantlets.

1 Materials and methods

1.1 Treatment of the first explants

Medium-sized (50 g) healthy bulb scales of *Lilium longiflorum*, stored in dark at 4℃ for 69–90 days to accomplish their vernalization, were used as the first explants to induce shoots. Each bulb contained 10–15 bulb scales. After washing in running tap water for 12 hours, bulb scales were sterilized on surface in 75% ethanol for 30 seconds, then sterilized in 0.1% $HgCl_2$ for 15 minutes and then washed in deionized water for three times. Bulb scales were cut into 1 cm ×1 cm sections, each bulb scale could be divided into 3–5 sections. These sections were cultured on shoot induction media.

1.2 Treatment of the second explants

Leaves, small bulbs and short basal stems which came from the shoots of first explants were cut into segments in size of 2 mm (width) ×10 mm (length) (leaves), 4–5 mm (width) × 7–8 mm (length) (small bulbs) and 8–10 mm (diameter) × 2 mm (height) (short basal stems). Each shoot could be divided into 15–18 segments. All these segments were used as the secondary explants and were cultured on shoot induction medium, too.

1.3 Culture condition

The shoot induction media was MS [16] supplemented with BA, 0.1 mg L^{-1} NAA (α-Naphthaleneacetic acid), 30 g L^{-1} sugar and 7 g L^{-1} agar. The concntration of BA was chosen from 0.5, 1.0, 1.2, 1.5, 1.8, and 2.0 mg L^{-1}.

The root induction medium was 1/2 MS containing 0.25 mg L^{-1} IBA (Indole -3-butyric acid).

All experiments were performed in petri dishes and glass bottles. Each vessel contained 15 ml medium. Media were adjusted to pH 5.8 before autoclaving at 121℃ for 15 min. Cultures for shoot induction and proliferation were performed under the light with 16 h photoperiod per day at quantum flux density of 50 μmol m^{-2} s^{-1} from fluorescent lamps. All cultures were incubated at 24 ± 2℃.

1.4 Low temperature treatment

After washing in running tap water, formed plantlets were treated at 4℃ for 0, 7 and 30 days, and then transferred to greenhouse at temperature of 28–30℃ (day) / 20–25℃ (night) and at illumination intensity of about 1 000 μmol m^{-2} s^{-1}.

1.5 Calculation of the number of plantlets

The total number of plantlets from one bulb could be calculated according to this formula: $T=s\times a\times b\times c\times d$

T: total plantlet number/bulb; s: the number of bulb scale/bulb; a: the segment number/bulb scale; b: shoot number/segment; c: segment number/shoot; d: shoot cluster number/segment.

2 Results

2.1 Shoot induction and proliferation

The original explant expanded quickly and formed shoots when cultured on the medium with different concentration of BA after 50 days. Considering the number and fresh weight of shoots induced, the MS medium containing 1.0 mg L^{-1} BA

was optimum for the formation and proliferation of shoots (Plate I: A; Table 1). The segments of leaves, small bulbs, and basal stems excised from the shoots arosed from the first explants were cultured in the same medium (MS medium containing 1.0 mg L^{-1} BA). Fourteen days later, the first batch of shoots from secondary explants formed (Plate I: B). Shoot clusters formed after 26 days. The size, number and fresh weight of shoots induced from the leaf segments were less than those of the shoots cluster generated from short basal stems. Considering the number and fresh weight of shoots, the sections of short basal stems were the best secondary explants for quick formation of shoots clusters, and followed by small bulbs (Plate I: C, Table 2).

Table 1 Effects of BA concentration on induction of shoots from bulb scales of *Lilium longiflorum* cultured for 50 days

BA (mg L^{-1})	Shoot number induced per bulb scale	Fresh weight of shoot per bulb scale (mg)
0.5	4.50±0.41 D	400.00±8.32 D
1.0	30.03±0.86 A	6000.00±89.90 A
1.2	24.03±0.84 B	4800.25±86.37 B
1.5	24.43±0.95 B	4856.75±75.69 B
1.8	23.60±0.71 B	4772.50±60.34 B
2.0	7.68±0.42 C	1126.00±3.65 C

Different letters in same column represent significant differences at P=0.01 by Ducan's multiple range test.

Table 2 Effects of different shoot sections on the formation of shoot clusters cultured for 40 days on MS medium containing 1.0 mg L^{-1} BA and 0.1 mg L^{-1} NAA

Shoot section	Shoot number induced per section	Fresh weight of shoot per section (mg)
Leaf	0.75±0.64 C	106.25±87.50 C
Small bulb	2.95±0.60 B	427.50±100.46 B
Short basal stem	8.68±0.82 A	1783.75±123.98 A

Different letters in same column represent significant differences at P=0.01 by Ducan's multiple range test.

2.2 Rooting and flowering

Shoots were excised from shoot clusters and were cultured on 1/2 MS medium supplemented with 0.25 mg L^{-1} IBA to induce roots (Plate I: D). Thirty days later, strong roots formed from shoots. After low temperature treatment as mentioned above, all the plants within 4 months stepped into flowering stage at the same time (Plate I: E). This result indicated that there was little effect of cold treatment time on flowering time.

2.3 Number of plantlets

In this study, number of plantlets could be calculated according to this formula: $T=s\times a\times b\times c\times d$, in which $s=10$, $a=3$, $b=30$, $c=15$, $d=4$. Considering the survival rate of 100% (data not shown), each bulb could produce about 54 000 new plantlets. It is suggested that the micropropagation efficiency of *Lilium longiflorum* had been greatly improved.

3 Discussion

Han et al.[2] reported the cycle method from shoot section to shoot cluster could effectively promote shoot proliferation. Similar results were also reported by Stanilova et al.[17], Maesato et al.[18] and Nhut[19]. We have confirmed that the two-step explant method worked efficiently, and the segments of short basal stems had the best regeneration capacity in *Lilium longiflorum*. The total number of plantlets from one bulb was about 54 000.

Different kinds of cytokinins have different effect on the shoot formation. Han et al.[2] reported that BA with IAA was more favorable on shoot formation. Our result had different from it. According to our research, when the BA concentration was 1.0 mg L^{-1}, the shoots were very strong and the number of shoots increased greatly (Table 1, Plate I: A, C). Therefore, we only used the same kind of medium of MS containing 1.0 mg L^{-1} BA and 0.1 mg L^{-1} NAA which was most suitable to initiate shoot and proliferate shoot (Table 1). Thus the whole process could be greatly simplified.

Lilium is one of the three bulb crops in the commercial market[1]. To ensure flowering, it requires vernalization. Han et al.[2,6] reported that the formed

bulblets generated flower stems after cold treatment at 5℃ for 2–3 months.

Ranwala et al.[20] reported that mature plants were stored in darkness at 3℃ for 2 weeks before placing them in a postharvest evaluation room (22℃) to accomplish vernalization. Therefore from bulb scale to flowering it required too long time, which greatly limited the contribution of flower to the market. According to our research, if the bulbs had been storaged at 4℃ to accomplish their vernalization, the new formed plantlets need not cold treatment at 4℃ before planting into greenhouse even in tropical area. Thus the growth period could be greatly shortened and only need 8 months. It might be due to all the cells of bulb which could differentiate into new plantlets had completed their vernalization during storage at 4℃ before culture. More interesting, even if the new formed plantlets had been treated at 4℃ for 0, 7, 30 days before culture, their flowering dates were the same in the first year (Plate I: E).

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Explanation of plate

Plate I:

A: The formation of shoots from bulb scales on MS medium +1.0 mg L^{-1}

BA and 0.1 mg L^{-1} NAA culture after 50 days;

B: The formation of the first batch of shoots from the sections of leaves, small bulb scales and short basal stems cultured on MS medium with 1.0 mg L^{-1} BA and 0.1 mg L^{-1} NAA for 14 days;

C: The formation of shoot clusters from the sections of short basal stems cultured on MS medium with 1.0 mg L^{-1} BA and 0.1 mg L^{-1} NAA after 40 days;

D: Root formation 1/2MS medium supplemented with 0.25 mg L^{-1} IBA cultured for 30 days;

E: Plants flowering after they are transplanted to soil for 4 months. a: without cold treatment; b: cold treatment at 4℃ for 7 days; c: cold treatment at 4℃ for 30 days.