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Studies on the Technique of Tissue Culture and Rapid Propagation of Bulbils From *Lilium regale*

Tao Lian, Deng Qun-Xian, Wang Yong-Qing, Liu Lu, Luo Shi-Feng,
Zhao Qing-Chun, Liu Jian-Xin and LV Xiu-Lan
College of Horticulture, Sichuan Agricultural University, Ya'an 625014, Sichuan, China

Abstract: Bulbils of *Lilium regale* were used as explants. Adventitious bud induction and the proliferation were studied. The results showed that the regeneration ability of outer layer scales of *Lilium regale* was better than the middle ones, which was better than the inner ones. The adaxial side of *Lilium regale* bulbils in contact with medium upward could produce more and stronger adventitious buds than the adaxial side downward. For outer and middle layer scales, the optimum medium for adventitious bud induction was MS +KT 1.0 mg L^{-1} + NAA 0.2 mg L^{-1} + CH 500 mg L^{-1}. The adventitious bud induction rate was 100.0% and each explant could produce 12.7 adventitious buds. For the inner ones, the appropriate medium for adventitious bud induction was MS + ZT 1.5 mg L^{-1} + CH 500 mg L^{-1} or MS + KT 1.5 mg L^{-1} + CH 500 mg L^{-1}. The medium MS + KT 0.5 mg L^{-1} + NAA 0.1 mg L^{-1} + CH 500 mg L^{-1} could make proliferation and rooting completed synchronously. The multiplication coefficient was 7.3.

Key words: *Lilium regale*, bulbils, tissue culture, rapid propagation, adventitious bud, *Liliaceae*

INTRODUCTION

Lilium regale, flowering bulb, is one of the most important species of *Liliaceae* and *Lilium*, which was discovered in Mingjiang region, Sichuan province by famous botanist Wilson E.H in 1903 and was introduced to Britain and America in 1905 and then became the worldwide cultivated variety (Yao *et al*., 2006).

In production, the common propagation method of Lily is division propagation. But the reproductive rate of division propagation is not only too low but also, the seedball is prone to degeneration due to accumulative diseases, giving rise to an adverse effect on growth and ornamental values (Zhao and Ma, 2007). Although, traditional asexual propagation like scale cutting is available for some lily varieties it is not frequently used because the scales are easily rotten after cutting (Qiu *et al*., 2004). Using tissue culture method can get not only high propagation coefficient, but also virus elimination and it can be also combined with genetic engineering and provide a new method for plant breeding (Li *et al*., 2008a).

Many researchers have studied culture *in vitro* of lilies (Kumar *et al*., 2006). Cultivated varieties were studied more and wild species were less focused on (Zhang *et al*., 2004), especially *Lilium regale*. Currently, there are only two cases of reports on culture *in vitro* of *Lilium regale* seeds (Sun *et al*., 2001) and bulbs (Xin and Luo, 2007). In most of previous researches, bulbs (Lian *et al*., 2003; Kumar *et al*., 2005), leaves (Bacchettal *et al*., 2003; Xu *et al*., 2009) and roots (Kapoor *et al*., 2008) have been used as explants, but the explants are limited to get and meanwhile a higher contamination rate is difficult to overcome. The bulbils are on the plant aerial parts, carrying less amount of germs, not easy to be damaged, seldom develop a phenomenon of endogenous bacterial in subculture (Guo and Lei, 2006). Moreover, bulbils of *Lilium regale* are enough in quantity, plump and easy to get. Picking bulbils does not affect not only the development of bulbs and flower bud differentiation, but also plant growth and flowering. Therefore, there are many advantages in using bulbils as explants.

Using bulbils as explants, the aim of the present study was to establish a more perfect *in vitro* regeneration system and provide theoretical and technical basis for rapid propagation, detoxification utilization and resource conservation of *Lilium regale*.

MATERIALS AND METHODS

The bulbils of *Lilium regale* were obtained from wild mature plants in Yaan. The experiments were carried out in the Comprehensive Laboratory SICAU China (Sichuan Agricultural University) (Fig. 1a).

Corresponding Author: Deng Qun-xian, College of Horticulture, Sichuan Agricultural University, Ya'an 625014, Sichuan, China

Adventitious bud induction: The bulbils of *Lilium regale* were rinsed with a little detergent and two drops Twain 20 and washed in running water for 1 h. Then they were surface sterilized with 75% alcohol for 20 sec first, rinsed twice in sterile distilled water and then sterilized with 0.1% $HgCl_2$ for 12 min followed by three final rinses in sterile distilled water. The bulbils were divided into three parts: the outer layer (outer 1~2 layers), the middle layer (outer 3~4 layers) and the inner layer (the rest). The scales were latitudinally cut for three times and inoculated by means of adaxial side upward and adaxial side downward. The medium was MS supplemented with NAA 0.2 mg L^{-1}, Casein Hydrolysate (CH) 500 mg L^{-1}, Sucrose 20 g L^{-1}, Agar 9 g L^{-1} and different concentrations of BA, KT and ZT, respectively. The PH was adjusted to 5.8. Data on explants forming adventitious bud were observed after 35 days.

Adventitious bud proliferation: Adventitious buds excised from *in vitro* scales were subcultured on MS medium containing BA (0.5, 1.0 and 2.0 mg L^{-1}), KT (1.0 and 1.5 mg L^{-1}) and NAA (0.1 and 0.2 mg L^{-1}). All the media contained kasein Hydrolysate (CH) 500 mg L^{-1}, Sucrose 20 g L^{-1}, Agar 9 g L^{-1} and were adjusted to pH 5.8. After 6 weeks of culture, the number and height of newly formed adventitious buds were recorded.

Culture conditions: In addition to the special statement, cultures were incubated at 25±2°C under 14 h photoperiod provided by white fluorescent lights.

RESULTS AND DISCUSSION

Effects of different parts of scales and ways of placement on adventitious bud induction: Table 1 shows that the regeneration ability of the outer layer scales of bulbil was better than the middle ones whose regeneration ability was better than the inner ones. The induction rate of the outer and the middle layer scales was higher (90.2 and 94.7%, respectively). The number of adventitious buds was more (9.4~11.1). By comparision, the induction rate of the inner layer was lower and the number of adventitious buds was less (71.5 and 2.3%, respectively). Besides, there were differences in contamination rate among three parts of scales. The sequence was the outer layer greater than the middle layer greayer than the inner layer.

That the adaxial side of *Lilium regale* bulbils in contact with medium upward produced a higher callus induction rate, differentiation rate,and larger number of adventitious buds than the adaxial side downward as shown in Table 2, the adventitious buds were rather sturdy, too (Fig. 1b and c). Therefore, under the same conditions, the layment of adaxial side upward was the optimum layment for adventitious bud induction and adventitious bud growth in bulbil scale culture *in vitro* in *Lilium regale*.

Effects of types and concentrations of cytokinin on adventitious bud induction: Three types of cytokinin (BA, KT and ZT) can induce the outer and middle layer scales of bulbils to form adventitious buds. From the callus induction rate and differentiation rate, it can be seen that KT produced the highest callus rate and differentiation rate, BA produced the lowest and ZT was in the middle. In terms of the number of adventitious buds, KT was better than BA, which was better than ZT. Higher concentration of BA and KT were better for adventitious bud induction. But the lower concentration of ZT enhanced adventitious bud induction.

Especially, the explants cultured on MS medium supplemented with 1.0 mg L^{-1} KT, 0.2 mg L^{-1} NAA and 500 mg L^{-1} CH exhibited the highest differentiation rate (100%), the largest number of adventitious buds (12.7) and the most sturdy adventitious buds as shown in Table 3.

By comparing differentiation rate and growth status of adventitious buds, it could be concluded that the suitable medium for adventitious bud induction of *Lilium regale* bulbil scales was MS basal medium added 1.0 mg L^{-1} KT, 0.2 mg L^{-1} NAA and 500 mg L^{-1} CH (Fig. 1d). The results showed that inductive effect of inner layer scales of bulbils of *Lilium regale* was worse than the outer and middle ones (Table 3 and 4). From the differentiation rate and number of adventitious buds, it

Table 1: Comparison of different parts of scales of bulbils in adventitious bud induction

Parts of scales	No. of explants	Contamination rate (%)	Differentiation rate (%)	No. of adventitious buds (cm)
The outer layer scales	78	34.4	94.7	11.1
The middle layer scales	61	15.4	90.2	9.4
The inner layer scales	76	5.1	71.5	2.3

Table 2: Effects of different ways of placing on adventitious buds induction

Placing way	No. of explants	Callus induction rate (%)	No. of calluses	Differentiation rate (%)	No. of adventitious buds	Length of adventitious buds (cm)
Adaxial side downward	56	71.9	7.9	71.9	8.2	0.4~2.0
Adaxial side upward	43	97.7	12.6	97.7	9.1	0.4~2.5

Table 3: Effects of types and concentrations of cytokinin on adventitious bud induction of outer and middle layer scales of bulbils from *Lilium regale*

BA (mg L^{-1})	KT (mg L^{-1})	ZT (mg L^{-1})	No. of explants	Callus induction rate (%)	No. of calluses	Differentiation rate (%)	No. of adventitious buds	Length of adventitious buds (cm)
1.0	0.0	0.0	66	84.20	9.10	86.40	8.00	0.7~1.2
1.5	0.0	0.0	62	87.50	14.8	87.50	12.7	1.5~1.7
0.0	1.0	0.0	68	100.0	14.6	100.0	12.7	2.5~2.6
0.0	1.5	0.0	63	100.0	9.90	100.0	10.9	0.4~0.8
0.0	0.0	1.0	64	95.80	10.3	95.80	11.3	0.8~2.0
0.0	0.0	1.5	66	90.80	5.80	90.80	10.2	0.2~1.2

Table 4: Effects of types and concentrations of cytokinin on adventitious bud induction of the inner layer scales of bulbils from *Lilium regale*

BA (mg L^{-1})	KT (mg L^{-1})	ZT (mg L^{-1})	No. of explants	Callus induction rate (%)	Differentiation rate (%)	No. of adventitious buds	Length of adventitious buds (cm)
1.0	0.0	0.0	28	0.0	50.0	1.0	0.4~0.5
1.5	0.0	0.0	27	10.3	63.0	1.0	0.4~0.5
0.0	1.0	0.0	26	12.5	92.3	1.0	0.5~0.6
0.0	1.5	0.0	22	31.8	81.8	6.0	0.3~0.4
0.0	0.0	1.0	24	12.5	66.7	1.0	2.0~3.0
0.0	0.0	1.5	24	26.7	80.0	8.6	0.3~0.6

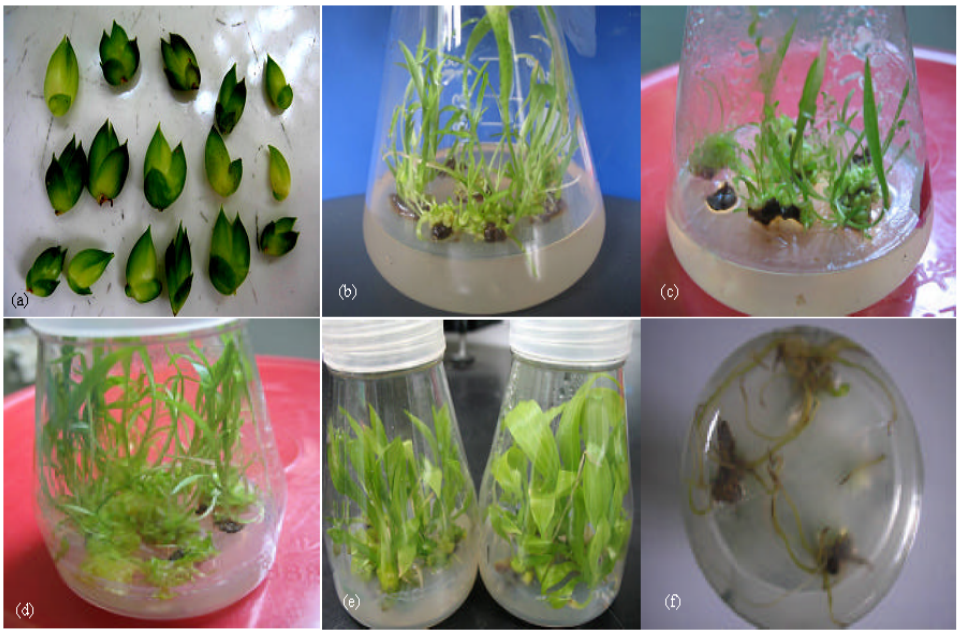

Fig. 1: a) *Lilium regale* 's bulbil; b-d) Adventitious bud induction; b) Adaxial side downward, MS medium supplemented with 1.0 mg L^{-1} BA, 0.2 mg L^{-1} NAA and 500 mg L^{-1} CH; c) Adaxial side upward, MS medium supplemented with 1.0 mg L^{-1} BA, 0.2 mg L^{-1} NAA and 500 mg L^{-1} CH; d) MS medium supplemented with 1.0 mg L^{-1} KT, 0.2 mg L^{-1} NAA and 500 mg L^{-1} CH; e-f):Proliferation: MS medium supplemented with 0.5 mg L^{-1} KT, 0.1 mg L^{-1} NAA and 500 mg L^{-1} CH

can be seen that 1.5 mg L^{-1} KT and 1.5 mg L^{-1} ZT exhibited the higher differentiation rate (80.0~81.8%) with larger number of adventitious buds per explants (6.0~8.6). Although, 1.0 mg L^{-1} BA exhibited the highest differentiation rate (92.3%), the number of adventitious buds was lowest (only 1.0) the appropriate medium for adventitious bud induction of inner bulbil scales from *Lilium regale* was MS basal medium supplemented with 1.5 mg L^{-1} ZT and 500 mg L^{-1} CH or MS basal medium supplemented with 1.5 mg L^{-1} KT and 500 mg L^{-1} CH.

Effects of BA, KT and NAA on multiplication in the multiplication cultures: Effects of different combinations of BA, KT and NAA on multiplication are shown in Table 5. The multiplication effects of different concentration combinations of KT and NAA were better than the combinations of BA and NAA. The multiplication coefficient (4.1~7.3) of the former was higher than the latter (1.22~3.4) and clumpy buds were sturdy. The medium supplemented with 0.5 mg L^{-1} KT, 0.1 mg L^{-1} NAA and 500 mg L^{-1} CH exhibited the largest

Table 5: Effects of BA, KT and NAA on multiplication in the multiplication culture

BA (mg L^{-1})	KT (mg L^{-1})	NAA (mg L^{-1})	No. of explants	The incidence rate of clumpy buds (%)	Multiplication coefficient (%)	Length of adventitious bud (cm)
0.5	0.0	0.1	62	41.9	1.6	3.0~6.0
0.5	0.0	0.2	63	57.1	1.9	2.5~6.0
1.0	0.0	0.1	92	15.2	1.2	3.0~6.0
1.0	0.0	0.2	71	39.4	1.7	2.0~4.0
2.0	0.0	0.1	71	71.7	3.4	2.0~4.0
2.0	0.0	0.2	79	53.2	2.7	2.0~5.0
0.0	0.5	0.1	66	74.2	7.3	4.0~6.5
0.0	0.5	0.2	78	70.5	4.4	4.0~5.5
0.0	1.0	0.1	57	66.7	4.1	3.0~6.0
0.0	1.0	0.2	51	76.5	5.4	5.0~6.0

multiplication coefficient (7.3), the highest incidence rate of clump buds (74.24%) and the clumpy buds grew strongly (Fig. 1e). It could be concluded that MS supplemented with 0.5 mg L^{-1} KT, 0.1 mg L^{-1} NAA and 500 mg L^{-1} CH was the optimum medium for multiplication culture of bulbil scales of *Lilium regale*.

In addition, it was found that all the clumpy buds could develop 3~5 roots ranging 3~4 cm in length (Fig. 1e). The plantlets could be successfully transplanted after acclimatization without a rooting culture. Effects of different parts of bulbil scales and ways of placement on adventitious bud induction.

Effects of different parts of bulbil scales and ways of placement on adventitious bud induction: Wang *et al.* (2002) showed the sequence of regeneration ability of bulb was the outer layer scales greater than the middle layer scales greater than the inner layer scales, which was similar to the results obtained in this experiment. The time of greening of the outer and the middle layer scales was almost in the next 6~10 days. Besides, the outer and the middle layer scales developed more adventitious buds and a larger number of calluses. Therefore, the outer and the middle layer scales are better explants in bulbil scale culture *in vitro* in *Lilium regale*.

Affected by plant polarity, differentiation of bulbil scales was influenced by the way of placement on medium. The result showed that the adaxial side in contact with medium upward could induce more and stronger adventitious buds than the downward, which was in accord with the result gained by Li *et al.* (2006). The different regeneration ability of different parts of bulbil scales may be caused by different content of endogenous hormones and associated with different relative contents of growth promoting substances and growth inhibitors and controlled by balance of the two kinds of endogenous hormones (Jin *et al.*, 2005). It was speculated that there may be different contents of endogenous hormones in different parts of bulbil scales. It also, may have something to do with charactistics of physiology and biochemistry or different nutrient substances in bulbil scales.

Effects of plant growth regulators on induction and multiplication of bulbil scales of *Lilium regale*: In previous reports of plant tissue culture on lily, a combination BA and NAA was widely used for shoot induction (Tanaka *et al.*, 1991; Kumar *et al.*, 2007; Li *et al.*, 2008b) and proliferation (Ding *et al.*, 2001; Guo and Lei, 2006). There was no report on the comparison of induction effect of BA, KT and ZT on plant tissue culture in *Lilium*. Studies were made of the effects of BA, KT and ZT on adventitious bud induction of bulbil from *Lilium regale* in this experiment. The results showed that for the outer and middle layer scales, the higher BA concentration could induce adventitious buds efficiently.

But the lower concentration KT and ZT had a significant promotion on bulbil scale induction and growth, otherwise for the inner layer scales. BA had no significant promotion on bulbil scale's induction and growth and the higher KT and ZT concentration was better for adventitious bud induction. Therefore, it would be advisable to choose the situable kinds of cytokinin and concentration according to material parts on culture *in vitro* of lily. In particular, it was the first time to study effects of ZT on adventitious bud induction in the present study.

Compared with the previous researches in which KT was used in combination with NAA for shoot proliferation of *Lilium regale's* bulbil scales. More strong plantlets with higher multiplication coefficient than combination of BA and NAA were obtained in the present experiment.

It was found that the clump buds from all treatments demonstrated etiolation in different degree and finally died (death rate ranging from 3.22-50.77%), when subculture time was over 60 days, especially in the treatment with higher multiplication coefficient. The main reason may be that the subculture time was so long that the medium can't meet clump buds nutrient requirement. In addition, the more clump buds the greater respiratory

intensity and the more metabolic substances produced by respiration. The experiment showed that the optimum subculture time of bulbil scales of *Lilium regale* was 45 days.

It was also found that all of the shoots cultured on the proliferation media developed a number of sturdy and good quality roots. These rooted plants could be transplanted after acclimatization without rooting culture, which would be of a practical significant importance in promoting industrial seedings of *Lilium regale*.

CONCLUSION

Bulbils from *Lilium regale* was used as explants. A tissue culture and rapid propagation system was established in this experiment, which would provide theoretical and technical basis for rapid propagation, detoxification utilization and resource conservation of *Lilium regale* and also, would be of a practical significant importance in promoting industrial seedings of *Lilium regale*.

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