Plant Micropropagation from *in vitro* cultured bulb scales of *Lilium lancifolium*

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Abstract: *Lilium lancifolium* Thunb. is one of the best lilies which are edible in China but the efficient shoot regeneration system has not been developed. The purpose of the present study is to establish an efficient and reproducible protocol for induction of shoots in vitro from *L. lancifolium* bulb scales Shoot regeneration from in vitro culture scales of *L. lancifolium* was tested on a orthogonal test with 4 factors and 3 level, containing different concentrations of BA–6-benzylaminopurine (6-BA) and a-naphthaleneacetic acid (NAA) and sucrose. The best adventitious shoot induction was showed when grown on media containing MS (Murashige and Skoog, 1962) +1.5mg/ L 6-BA and 0.1 mg/L NAA+20g/L sucrose according to the result. The highest adventitious shoot proliferation multiple (4.1) was observed when grown on media containing1.0 mg/ L 6-BA and 0.1 mg/L NAA. Regenerated shoots were rooted after 20 days grown on media containing 1/2MS+ 0.4mg/L NAA.
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1. Introduction

The genus *Lilium*, with approximately 220 species, belongs to the large family of the Liliaceae. At present, 80 species of *Lilium* are found in the temperate and subtropical zones of the northern hemisphere (Woodcock and Stearn 1950; Feldmaier and McRae, 1982) and 47 species 18 varieties of *Lilium* are mainly for the edible and medicinal use. Conventional breeding in the lily is hampered by its heterozygous state and self -incompatibility among the species of the different *Lilium* groups (Van Tuyl et al., 1990). The development of efficient systems for transformation could accelerate the breeding process, but these techniques require an efficient and reproducible protocol for the induction of adventitious shoots. In lily, plant regeneration has been achieved from a vast array of explants ranging from flower organs to bulb scales (Han et al., 1997; Mitsuguet al., 2002; Kumar et al., 2005).

Tissue culture techniques have been successful for rapid propagation of some members of the genus Lilium, including *L. Iongiflorum* (Nhut, 1998), *L. rubellum* (Nimii et al., 1997), *L. lancifolium* (Marinangeli and Curvetto, 1997), *L. auratum* (Takayama and Misawa, 1979, 1980, 1983), and *L. testaceum* (Wozniewski et al., 1991). However, there have been few reports about micropropagation of *L. lancifolium* where bulb scales originating from regenerants through in-vitro culture are used.

L. Lancifolium known as DaoChui lotus, tiger lily, is a perennial herb, as the Liliaceae Lilium of bulbous perennial flowers. Along with the development of the economy, the lily cut flower production will become the mainstreams and it needs a lot of ball. At present, it is not able to meet the needs of edible production. The lily bulb is mainly for vegetative propagation, breeding bulb quantity easily degenerated and their breeding cycle is longer. Tissue culture can not only solve the degradation of the lilies but also can solve the problem of detoxification and propagation.

The objective of the present study was to establish cultural conditions where shoot organogenesis from bulb scales could be improved. Furthermore, the results of micropropagation that used bulb scales segments are described.

2. Materials and methods

Plant material

Primary explants consisted of the bulb scales of the *L. lancifolium*. Bulbus were washed thoroughly under running tap water for 2 hours, and then submerged in 70% ethanol for 20s. They were then rinsed three to five times with distilled water and placed in a 0.1%(wt/vol) aqueous solution of $HgCl_2$ for 13 min and rinsed three to five times in sterile distilled water. Under aseptic conditions, the bulb scales were excised from the bulbs and the lower portion (0.5 cm × 0.5 cm) of the inner scales was used as the explants.

Culture conditions

Pseudo-bulblet induction culture

The explants were inoculated on MS containing different concentrations of hormone and sucrose. At first, with a L 9 (3^3) orthogonal designed experiment we established the optimum association of 6-BA, NAA and sucrose with two bulb scales per glass jar and 10 replications (glass jars) for each treatment(Table 1). The total induced pseudo-bulblet number of *L. longiflorum* was recorded.

Table1. Orthogonal test with3 factors and 3 levels L 9(3^3) for induction of shoots from *L. lancifolium* bulb scales

6-BA (mg/L)	NAA (mg/L)	sucrose (g/L)
1.0	0.1	10
1.5	0.2	20
2.0	0.3	30

Adventitious bud proliferation culture

The induced pseudo-bulblets were excised when they reached a size of 2–3cm and transferred onto MS medium supplemented with different hormone concentrations for adventitious bud proliferation. The adventitious bud proliferation experiment was also set up in a completely randomized block design with two pseudo-bulblets per glass jar and 10 replications (glass jars) for each treatment.The induced adventitious buds were placed with medium onto the following media:

1 MS+0.5 mg /L 6- BA+ 0.1mg /L NAA
2 MS+1.0 mg /L6- BA+ 0.1mg /L NAA
3 MS+1.0 mg /L 6- BA+ 0.2mg /L NAA
4 MS+1.5 mg /L 6- BA+ 0.1mg /L NAA

Rooting culture

For the rooting, the shoots (about 3cm in length) were excised, and placed vertically into half-strength MS basal medium containing four NAA concentrations (0.2, 0.4, 0.6 and 0.8 mg/L) and 15 g /L sucrose, solidified with 7 g /L agar. The rooting experiment was also set up in a completely randomized block design with two shoots per glass jar and 10 replications (glass jars) for each treatment.

Medium was adjusted to pH 5.8 before autoclaving at 121 ℃, 1 atm for 30 min. Cultures were incubated at 25±1℃ with a photoperiod of 12 h per day at a light density of 40umol m^{-2} s^{-1} of (in light condition) and 70–80% relative humidity.

3. Results and discussion

pseudo-bulblet induction culture

L. lancifolium bulb scales was cultured on the nine kinds of media with different concentrations of hormone and sucrose, after 7 days of incubation, most bulb scale explants of *L. lancifolium* showed elongation and enlargement. Regenerated shoots appeared within 7-11 days of culture initiation. While they resembled bulblets morphologically, they did not exhibit dormancy or an arrested phase in their development. Bulb scales gradually turned into green and bulb scales have obviously thicken at the same time, pale green embryoid was the first appeared in turned green bulb scales, then the adventitious bud was directly induced by the embryoid .When the callus differentiation particles and adventitious bud were not induced, the bulb scale explants gradually turned into browning and eventually died. The detailed orthogonal experiments and related results are listed in Tables 2 respectively. By variance analysis, the order of influence for induction adventitious buds is BA–6-benzylaminopurine (6-BA), a-naphthaleneacetic acid (NAA) and sucrose.The optimum experiment conditions is: 1.5 mg/L6-BA, 0.2mg/L NAA and 20g sucrose(*Figure 1)*.

Table 2 Effect of various combinations of BA–6-benzylaminopurine (6-BA), a-naphthaleneacetic acid (NAA) and sucrose on pseudo-bulblet induction from bulb scales of *L. lancifolium*

Treatments	6-BA *(mg/L)*	NAA *(mg/L)*	sucrose*(g/L)*	Total number of adventitious buds	The time of turning green
T1	1.0	0.1	10	82	7
T2	1.0	0.2	20	80	8
T3	1.0	0.3	30	77	10
T4	1.5	0.1	30	79	9
T5	1.5	0.2	10	86	8
T6	1.5	0.3	20	82	7
T7	2.0	0.1	20	66	9
T8	2.0	0.2	30	72	9
T9	2.0	0.3	10	64	12
F	109.8	11.48	8.1		

Adventitious bud proliferation culture

Within 40 days, each pseudo-bulblet developed into a rosette of shoots. The number of shoots on each rosette varied with the amount of 6-BA and NAA. The best proliferation medium was MS +6-BA 1.0mg/L+NAA 0.1mg/L, in which the average number of shoots can reach 4.1 and length 1.8 cm (Figure 3 .Table3).

Table 3 The effect of 6-BA and NAA on the number of adventitious bud proliferation from pseudo-bulblet

Treatment	6-BA *(mg/L)*	NAA *(mg/L)*	The total number of adventitious buds proliferation from 10 bottle of pseudo-bulblets	Mean number of shoots per pseudo-bulblet
P1	0.5	0.1	63	2.1±0.1
P2	1.0	0.1	123	4.1±0.1
P3	1.0	0.2	111	3.7±0.1
P4	1.5	0.1	93	3.1±0.1

Rooting culture

When transferred to half MS medium supplemented with different concentration NAA, all of treatments used in the present study induced rooting. However, the improvement of rooting was found in half-strength MS medium supplemented with 0.2–0.8 mg /L NAA (Figure 4 .Table4).

Table 4 Effect of NAA on rooting of *L. Lancifolium* regenerated shoots after 20 days of culture

Treatments	*NAA(mg/L)*	*Number of roots per shoot*
R1	*0.2*	*3.78* ±0.78
R2	*0.4*	5.94 ±2.34
R3	*0.6*	3.80 ±0.39
R4	*0.8*	2.78 ±0.99

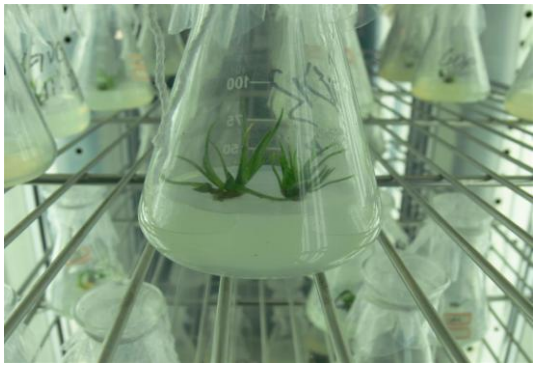

Figure 1 Pseudo-bulblet of *L. lancifolium* derived vitro-grown sprout of bulb

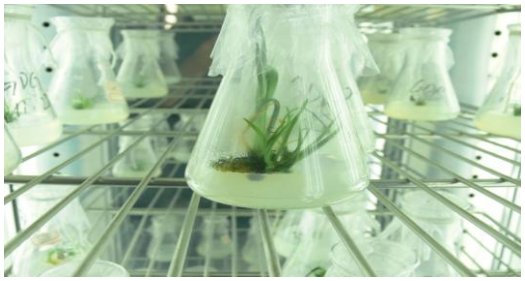

Figure 2 callus of *L. lancifolium* derived from in from in vitro-grown sprout of bulb.

Figure 3 Multiple shoots developing from one pseudo-bulblet cultured in MS medium

Figure 4 elongated shoots rooted after20 days o f culturing

4. Discussion

In this experiment, applications of middle levels of BA–6-benzylaminopurine (6-BA) can enhance meristem formation and shoot proliferation and may manifest results similar to those caused by cytokinins in many species (Reynolds, 1987). Likewise, Yun et al. (2000), studying the influence of 6-BA, a-naphthaleneacetic acid (NAA) and sucrose on the development of *L. lancifolium*, found that 6-BA was the most effective in promoting regeneration. We examined their effects on shoot induction from bulb scale explants, and found that organogenesis depended on the specific type of plant growth regulator used in the MS media. Several studies have demonstrated that BA is a very effective promoter of shoot induction and multiplication from Liliaceae (Godo et al., 1998; Nhut, 1998; Ulrich et al., 1999). Although plants treated with higher levels of BA produced more shoots, they were very short and compact, and some had abnormal leaves. In contrast, lower concentrations may have little effects on shoot growth (Ulrich etal., 1999; Han et al., 2001).

The pseudo-bulblets occurred from the bulb scales accompanying with a few calluses in some medium (Figure 2). The best pseudo-bulblet induction medium designing the orthogonal test and the optimum experiment conditions is MS +1.5mg/L 6-BA +0.2mg/L NAA+20g/L sucrose. A rosette of shoot arised from the pseudo-bulblet. The best adventitious bud proliferation medium is MS +1.0mg/L 6-BA +0.1mg/L NAA and the highest proliferation multiple is 4.1. Regenerated shoots have been rooted and the best rooting medium is 1/2MS +0.4mg/LNAA.

This research demonstrates the excellent regeneration capacity for *L. lancifolium*. Our success with in-vitro establishment clearly indicates that micropropagation is an effective and useful technique for the reproduction of this species. Mass production

via tissue culture may also become a desirable alternative to seed propagation, since the latter method has inherently low germination rates and a slow growth process.The orthogonal test is a scientific and systematic design method by which concise test set with fewer test cases is created. Another advantage of the orthogonal test, compared to all factorial design, is reduction of testing treatment amounts and determination of scientific results (Zheng et al., 2010). In this orthogonal test, only 9 treatments were performed compared to 71 treatments in all factorial experimental design, which consumes more costs.

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