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Bulblet formation and development of Lanzhou lily (*Lilium davidii* var. *unicolor*) by tissue culture

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Abstract: Bulblet formation and development of Lanzhou lily (*Lilium davidii* var. *unicolor*) by tissue culture could provide technical solutions to realize the mass production of Lanzhou lily bulblet, which needs three steps to achieve, including multiple shoots proliferation, induction of bulblets and enhancement growth of bulblets. The starch content and the characteristic parameters of bulblet were detected at different culture stages. This study acquired an advanced technique that could effectively promote the bulblet formation and development, and induce the growth of main stem. The results demonstrated that the bulblet diameter, weight and number of scales were up to 1.66 cm, 2.48 g and 26.33 pieces, respectively. The starch content showed a gradual increasing trend according to the culture process, which indicated that the starch content was positively correlated to the development of bulblet. In addition, the bulblet size, weight and number of scales showed a positive correlation. The growing point of bulblet was easy to form the main stem when the number of scales reached to 26 or more. In this paper, the invented three-step tissue culture technology effectively promotes bulblet enhancement development, and enlargement of the bulblet can effectively shorten the field growth cycle, improve lily production on time, and also, it can provide technical reference for achievement of mass production of enhancement bulblet.

Key words: *Lilium davidii* var. *unicolor*, bulblet, development, starch, plant hormone

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兰州百合组培鳞茎发育研究

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摘 要: 该研究以兰州百合商品种球鳞片为外植体材料,通过组织培养诱导丛生芽萌发及高频增殖,再以丛生芽为材料诱导其发育形成小鳞茎,调节培养基对小鳞茎进行膨大发育培养,最终形成促进兰州百合组培鳞茎膨大发育的"三步"组培培养技术路线;对发育过程中形成的丛生芽、小鳞茎及膨大鳞茎进行淀粉含量测定与生长特征参数统计,分析各步培养对鳞茎形成发育过程中淀粉含量与形态变化的影响。结果表明:所建立的"三步"培养方案培育出的组培鳞茎直径、重量与鳞片数分别为1.66 cm、2.48 g和26.33片,有效地促进了鳞茎的膨大,并能诱导鳞茎主茎杆的形成发育;在培养进程中其淀粉含量呈现逐步增加的趋势,这表明与鳞茎膨大发育正相关,同时鳞茎大小、重量及鳞片数三者也表现为正相关性;当鳞茎所含鳞片数在26片以上时,其生长点易发育形成主茎杆。该文研究了兰州百合组培鳞茎的形成与膨大发育技术,所研发的"三步"培养组成的鳞茎膨大发育组培

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技术有效地促进了鳞茎的膨大发育,而膨大发育的鳞茎能有效地缩短田间生长周期,从时间上提高百合生产量,同时为实现兰州百合膨大的鳞茎种球规模化生产提供技术支撑。

关键词:兰州百合,小鳞茎,发育,淀粉,植物激素

Lanzhou lily, a variation of *Lilium davidii*, is the main edible and the only sweet lily in China, which has big bulb, gorgeous color, high quality and medicinal value. Lanzhou lily has broad marketing space and developing prospect because of its advantages. However, it has a long natural growth period, and the conventional propagation pattern is to sow scales that possess low-cost and handleability. The propagated bulblet can not develop main stem in the first year, which only grows some basal leaves for growth of the bulblet (Gao et al, 1986). It needs 2-3 a for formation of the big bulblet with developed main stem which can be transplanted directly as seed-bulb (Teng & Song, 2005). The commercial bulb would be harvested after the seed-bulb with main stem had transplanted and grown for another 2-3 a (Wang, 2010).

In this connection, the development of bulblet and formation of main stems by tissue culture are of great significance in commercial production of Lanzhou lily bulblet. The research about tissue culture of Lanzhou lily focuses on induction of regeneration plant, for example, inducing sprouting and plant regeneration from Lanzhou lily scale and leaf explants (Long et al, 2004; Han & Guo, 2009), inducing callus formation and shoot differentiation from petals (Liu, 2007), but there is little on the bulblet formation and development of Lanzhou lily. It has been reported that high concentrations of glucose could effectively promote the bulblet formation and development of other lilium plants (Kumar et al, 2005; Varshney et al, 2000; Rice et al, 1983; Taeb & Alderson, 1990). Nhut (2003) reported that the combination of N^6-benzyladenine (BA) and gibberellic acid (GA_3) could promote main stem formation directly from the *in vitro* receptacle of *Lilium longiflorum*. Up to now, there is no report about the bulblet formation and development of Lanzhou lily. To overcome low proliferation rates and long period of Lanzhou lily bulblet propagation, in this study, the propagation techniques of tissue culture were used to cultivate bulblet of Lanzhou lily at different stages, and to improve its bulblet formation and development. The correlations between starch contents and characteristic parameters of bulblet growth were investigated. The changes of characteristic parameters of bulblet swelling, especially the parameters changes of main stem formation were also examined. The proposed technology on bulblet formation and development of Lanzhou lily was establishment, which would lay the foundation for large-scale production of bulblet.

1 Materials and Methods

1.1 Experimental materials

Tissue culture plantlets of Lanzhou lily were provided by Biological Technology Institute of Guangxi Academy of Agricultural Sciences. The media were set as, Fp1: MS+0.3-0.5 mg · L^{-1}BA+0.03 mg · L^{-1}NAA + 30 g · L^{-1} Sucrose+5 g · L^{-1}Agar; Fp2: MS + 90 g · L^{-1} Sucrose+5 g · L^{-1}Agar; Fp3: MS+0.15 mg · L^{-1}BA+0.15 mg · $L^{-1}GA_3$+ 90 g · L^{-1} Sucrose+5 g · L^{-1}Agar.

1.2 Experimental methods

1.2.1 Culture approach The whole culture process was comprised of three steps. Step 1, Fp1 medium was used to culture multiple shoots of Lanzhou lily. Steps 2, the leaves of multiple shoots were excised and transferred the multiple shoots to Fp2 medium for two subcultures. Step 3, the basal leaves of bulblets on Fp2 medium for one subculture were excised and transferred the multiple bulblets to Fp3 medium for two subcultures. The growth status in every step was recorded. All culture conditions as follow, (25±2)℃ with a light intensity of 150 μmol · m^{-2} · s^{-1} and 14 h light/10 h dark photoperiod for 28-30 d of one subculture period.

1.2.2 Trial on the change of culture step The multiple shoots of Lanzhou lily cultured on Fp1 transferred into Fp3 medium directly without inducing bulblet on Fp2. The growth was recorded.

1.2.3 Starch content determination The starch content was estimated following the spectrophotometric method according to Liu et al(2013).

1.2.4 Material preparation and electron microscopy observation　Observation of cultures at different development phases through scanning electron microscope (SEM) has been done. The basal portion of adventitious shoot from step1 and scales of bulblets from other step were observed with SEM (Tescan, VEGA Ⅱ LUM, Czech), and the treatment method for specimens refer to Nhut (2003).

1.3 Data analysis

Each treatment had 30 explants and was repeated 3 times. Explants in experiments were arranged in a completely randomized design. Data were presented as mean ± standard error of three independent experiments, and was analyzed for significance by analysis of variance with the mean separation by Duncan's multiple range test and significance was determined at 5% level (SPSS 15.0).

2　Results and Analysis

2.1 Culture approach of Lanzhou lily bulblet formation and development

Multiple shoots could propagate stablely without vitrification through Step 1 (Fig. 1: a), and the proliferation coefficient got to 3－4. The bulblets formed in Step 2 and the bottom of leaves enlarged and formed small scales with significant differences in leaf morphology. The whole base of buds swelled gradually into an oval shape to achieve a change from buds to bulblet (Fig. 1: b). Step 3 was the enhancement and development of bulblets, in which the scale enlarged and the bulblet size increased rapidly (Fig. 1: c). The transformation of tissue culture plantlet of Lanzhou lily from multiple shoots to bulblets was achieved according to the three continuous culture steps.

The growth of bulblet was explored by changing the culture approach. As shown in Fig. 1: d, the multiple shoots failed to form bulblet and remained the multiple shoot status after the multiple shoots obtained from Step 1 transferred to Step 3 directly for one subculture.

The increase of bulblet size was much more through twice subculture than once subculture in Step 2 (Fig. 1: e, Table 1), with the increase rates of diameter and weight were 33.33% and 80.08%, respectively, but the increase was significant less than that in Step 3 (Table 1). Compared to the first subculture in Step 2, the increase rates of diameter and weight were up to 180.56% and 894.29% in Step 3 for one subculture. The bulblet grew more rapidly after culture for two cycles in Step 3, with the diameter and weight of bublet were up to 1.32 cm and 1.66 g (Table 1). Some of the bulblets developed main stems in this stage (Fig. 1: f).

Table 1　Formation development of bulblets in Step 2 and enhancement growth of bulblets in Step 3

Culture phase	Bulblet diameter (cm)	Bulblet mass (mg)
Once subculture in Step 2	0.36±0.02 d	87.26±3.35 d
Twice subculture in Step 2	0.48±0.05 c	157.14±5.78 c
Once subculture in Step 3	1.01±0.03 b	867.62±4.39 b
Twice subculture in Step 3	1.32±0.05 a	1663.28±8.17 a

Note: Values are means ± SE. Means in a column followed by the same letters are not significantly different according to Duncan's multiple range test at the 5% level. The same below.

2.2 Starch content change of Lanzhou lily during the bulblet formation and development

The starch contents of Lanzhou lily in the process of bulblets formation and development were estimated (Table 2). The starch content was the lowest with the medium containing 30 g · L^{-1} sugar in Step 1. However, the starch content increased significantly ($P<0.05$) and up to 32.54% through increasing the sugar concentration to enhance the bulblets formation in the Step 2. By adding the BA, GA_3 and sugar to stimulate the bulblets formation, the starch content of bulblets increased to 42.68% in Step 3. Compared to Step 1 and 2, the starch contents increased 113.83%, 31.16%, respectively. The results demonstrated that the starch content increased gradually in the process of tissue culture Lanzhou lily bulbets formation and development from multiple shoots induction, and the starch content was positively correlated to the development of bulblet.

The starch grain formation during the development of multiple shoots and bulblets was oberved with scanning electron microscope (SEM), as shown in (Fig. 2: a). The bulblets starch grain increased obviously on the condition of bulblets induction and development (Fig. 2: b, c).

2.3 Correlation between main stem formation and bulblet diameter, weight and scale number

The development of main stem was promoted with

Table 2 Starch content change during the development process of Lanzhou lily

Developmental stage	Starch content (%)
Multiple shoots (Step 1)	19.96±0.31 c
Bulblet formation (Step 2)	32.54±0.18 b
Enhancement growth (Step 3)	42.68±0.43 a

the increasing of bulblet size and weight in the process of Lanzhou lily tissue culture bulblet development. However, in its natural breeding process, it needs 1–2 a from planting scales to forming bulblets with main stem (Gao et al, 1986). Therefore, it is of great importance for bulblet production that Lanzhou lily bublet development and main

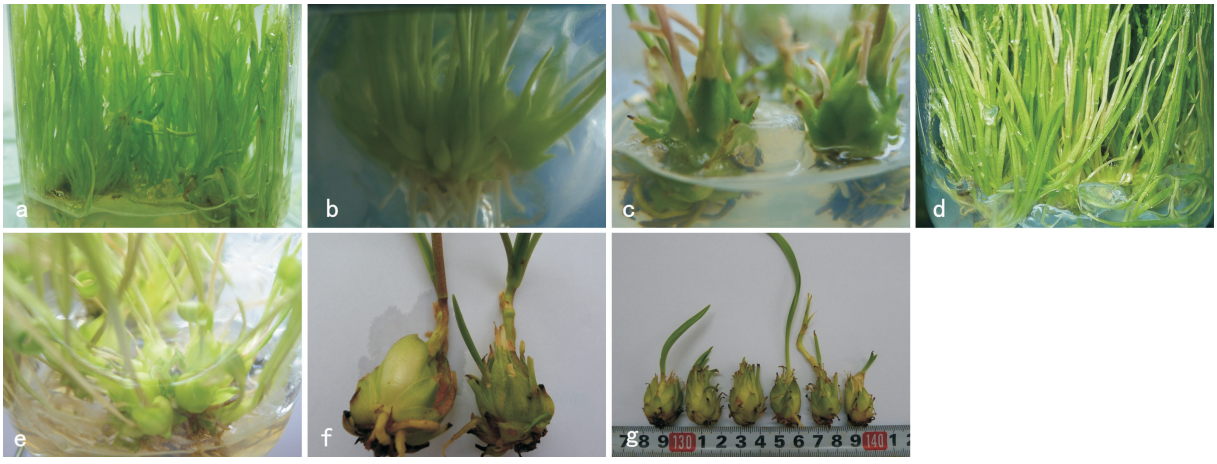

Fig. 1 Efficient approach of inducing bulblet development using bulb scale explants of Lanzhou lily **a**. Multiple shoots induced from callus in Step 1; **b**. Little bulblets formation on Fp2 in Step 2; **c**. Bulblets growth and development on Fp3 in Step 3; **d**. Shoots development come from the approach of Step1 to Step 3 directly without Step 2 of little bulblet formation culture; **e**. Bulblets development on Fp2 in Step 2 for twice subculture; **f**. Bulblet main stem formation in Step 3; **g**. Obtained big bulblets in Step 3.

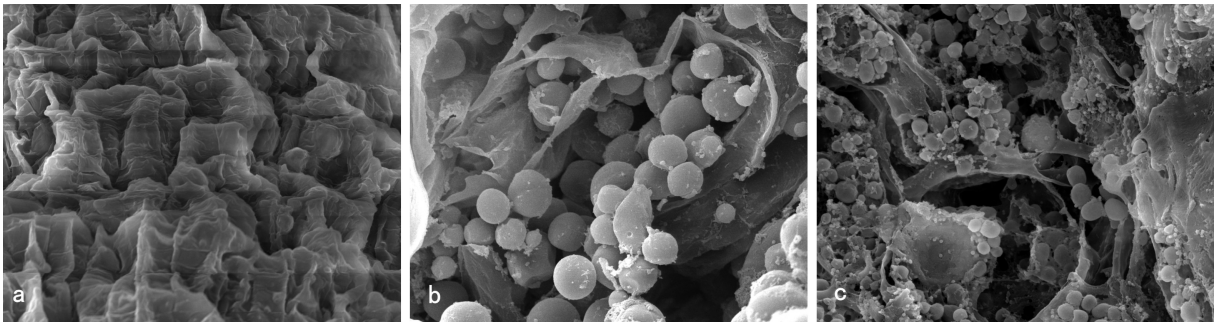

Fig. 2 SEM allowed clear visualization of starch grain formation during Step 1–3. **a.** Transverse section of shoot's base in Step 1, hardly to find starch grain; **b**, **c**. Plenty of starch grain formed on the inner of scales in turn in Step 2 and Step 3.

stem formation by tissue culture. The parameters of bublets such as diameter, weight, number of scales, were investigated in the once and twice subculture in Step 3 (Table 3). The data indicated that the bulblet diameter, weight and number of scales, which formed the main stem, were significant higher than that did not form main stems ($P<0.05$), with 22.96%, 48.50% and 38.36% increased rate, respectively. Meanwhile, it was easy to form main stems while the scale number of bulblets reached to 26 or more.

As shown in Fig. 3, the bulblet diameter, weight

Table 3 Comparison of bulblets that formed and unformed the main stem

Bulblet type	Diameter (cm)	Mass (g)	Number of scales per bulblet
Unformed main stem	1.35± 0.14 b	1.67± 0.16 b	19.03± 1.61 b
Formed main stem	1.66± 0.12 a	2.48± 0.15 a	26.33± 2.43 a

and number of scales showed a positive correlation between each other. The increasing of bulbets size accompanied with the increasing of weight and scale number during the bulblets development.

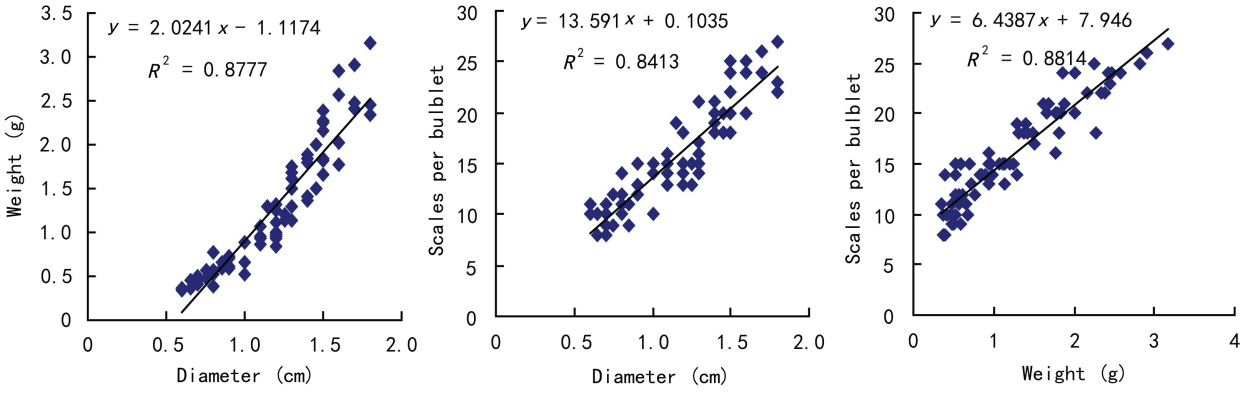

Fig. 3　Relativity of bulblet diameter, weight and scale number during the bulblet development

3　Discussion

There are some related studies focused on tissue culture techniques about Lanzhou lily buds development and multiple shoots propagation inducing by the scale explants (Han & Guo, 2009; Liu et al, 2010). In this study, combination of certain concentration of BA and α-naphthaleneacetic acid (NAA) that have been reported by former studies (Lin et al, 2008; Xu et al, 2009), were used to propagate multiple shoots stably (Fig. 1: a). However, these researches were limited to the regeneration shoots by tissue culture, lack of the bulblet development research. Based on the proliferation of multiple shoots culture for the first phase of Lanzhou lily bulblet development, it provided bud materials for the formation and enlargement growth of bulblet. However, the bulbs formation and development mainly proceed in the following Step 2-3, which was difficult to proliferate. The proliferation rate of the whole tissue culture system mainly reflected in the first stage of buds proliferation rate.

The bulblet size increased significantly (Fig. 1: e) through two successive subcultures in the Step 2, which indicated that the high concentration of sugar could continuously stimulate bulblet growth to a certain extent, at the same time, the quantity of bulblet formed was related to the multiple shoot size and growth state, in general bud block of 1 cm ×1 cm size could form 10 - 15 bulblets (Fig. 1: b).

Continuous twice subculture with a high concentration of sugar could promote bulblet growth in Step 2, but the growth rate still could not meet the requirements for the bulblet development. In order to make the bulb development more, the enhancement growth of bulblet in Step 3 has been used, the use of combination of BA and GA_3 promote bulblet enlargement development. As shown in Table 1, the bulblet diameter and the weight increased significantly ($P<0.05$) (Fig. 1: c) by the once subculture in Step 3 than by the twice subculture in Step 2, and increased at a greater rate through the twice subculture in Step 3, diameter and weight reached 1.32 cm and 1 663.28 mg, and could induce the formation of main stem (Fig. 1: f, g). The effects of continuous twice subculture in Step 2 could not achieve the effects in Step 3, so it showed the necessity of bulblet swelling growth culture in Step 3. It was reported that BA in combination with GA_3 could effectively promote the formation of main stem from longiflorum receptacle explants in the literature (Nhut, 2003), but the induction main stem of Lanzhou lily bulblet has not been reported. In our study, the BA and GA_3 with high concentration of sugar was of obviously positive significance for Lanzhou lily bulb expanded rapidly development and main stem formation.

By changing the culture approaches, the multiple shoots, which obtained from Step 1 turned to Step 3 directly, could not be induced to form bulblets but remains multiple shoots (Fig. 1: d). It showed that the materials used in Step 3 must be formed bulblets to promote its rapid enlargement and development. The bulblets development did not achieve the best through the continuous culture in Step 2. Through three steps, including reproduction by buds, inducing of small bulbs and swelling of bulbs, bulblets formation and development of Lanzhou lily by tissue culture could provide the best technical solu-

tions to realize the scale production of Lanzhou lily bulb development. Its main features included the first buds reproduction provided a higher multiplication factor buds to achieve stable growth, the multiple shoots formed small bulblets in Step 2, and the bulblets developed quickly to form bulbs with the main stem.

A physiological and biochemical change of carbohydrate metabolism is an important process in the development of lily bulb, the metabolism of energy storage and decomposition is the basis of lily morphology (Sun et al, 2008). This study showed that the starch content increased significantly in the buds proliferation, bulbs formation and bulbs swelling stage, which was positive correlated to bulbs development. A high concentration of sugar promoted the synthesis of starch, BA and GA_3 plant hormones effectively improved the conversion bulb sugar to starch. Meanwhile, the accumulation of starch promoted the bulbs enlargement. Electron microscope results similar to this, multiple shoots stage of Step 1 was not observed with starch grains, bulblet formation and developmental stages of Step 2-3, a great amount of starch grains have been synthesized (Fig. 2: a-c). Studies show that sugar and starch metabolisms play an important role in the regulation during the morphogenesis of Lanzhou lily bulblet.

The formation of main stem during the development of bulblet growth development has been analyzed for the first time. During the bulblet formation and enlargement of the development process, bulb diameter, weight and the number of scales showed positive correlation, and bulblet showed main stem pumped growth when a certain number of scales (more than 26) have formed. Gao (1986) reported Lanzhou lily growth point development could be divided into three stages, scale differentiation stage, apical bud differentiation phase, and flower bud differentiation stage. The main stem differentiation showed that growth point of bulblet began to change from scale differentiation to apical bud differentiation.

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