OPTIMISATION OF AN *IN VITRO* PROPAGATION PROTOCOL FOR A VALUABLE LILY (*Lilium spp.*)

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SUMMARY

Lily species have been used as ornamental plants for centuries. However, micropropagation of Lily *in vitro* depends on particular species. Therefore, in this study, a protocol for micro propagating Lily was optimized. The results indicated that *in vitro* type 1 Lily scales (near the basal stem) cultured on MS medium supplemented with 60 g/l saccharose, 0 g/l glucose, 0.5 mg/l BA, 0.1 mg/l NAA, 100 ml/l coconut water, 5.5 g/l agar and 1 g/l activated charcoal in full dark conditions is the best with the highest regeneration rate of bulbscale (3.68 bulblet/slice). *In vitro* Lily bulblets became healthy and bigger, formed roots in MS medium supplemented with 0.2 mg/l NAA. *In vitro* Lily bulblets were found unsurvival and ungerminated without pre-cold treatment. The cold treatment time can vary between 4, 6, and 8 or 10 weeks, that did not affect the plant height and leaf numbers. The studies also found that the weight of bulblets significant affect plantlet height and leaf numbers.

Keywords: *In vitro*, *Lilium* spp., Lily micropropagation, tissue culture.

I. INTRODUCTION

The lily (*Lilium* spp.) is a well known genus as one of the most beautiful flower species. Today they are important plants that are grown in gardens and cultivated for cut flowers and have become economically important in the flower industry. Tissue culture has been applied to propagate Lily since the late 1950's (Robb, 1957). Lily tissues in general have a high regeneration potential and bulb scales have the best capacity to regenerate adventitious bulbs. Hence, bulb scales are most commonly used as explants for traditional vegetative propagation. Unfortunately, being under-ground parts, there is a high contamination risk with bulb scales. Mass production and fast regeneration of uniform plant material in tissue culture is a necessity for the breeding and culture of lilies. However, to make tissue culture a commercially relevant production system, production protocols need to be developed separately for each plant crop and cultivar.

The contribution of phytohormones on the morphogenesis of differentiating Lily plants has been studied in various respects. The presentation of auxin, α-naphtalene acetic acid (NAA) and cytokinin (kinetin) found essential in the formation of bulblets and roots; higher auxin/cytokinin ratio increased root formation whereas lower ratio increased bulb formation (Takayama and Misawa, 1979). When different cytokinins, such as 6-benzyladenine (BA), kinetin, 2iP and zeatin, were tested in combination with NAA, differences in regeneration response in general were found (Maesato et al., 1994). Besides NAA, Ruffoni, B., Mascarello, C. and Savona, M. (2010) reported that a combination of NAA and BA gave the best differentiation response. In this study, culture mediums were tested to find a suitalbe commercially relevant production system for Lily.

II. RESEARCH METHODOLOGY

1. Material

OT hybrid Lily imported from Holand with 1.5 - 2 mm Lily bulbscale sildes or 0.5 - 4 g bulblet were used as initial explants as described by Bui Thi Thu Huong *et al*., (2013).

2. Method

a. Investigation of different nutrients and phytohormone on platn regeneration:

The cultivation medium used to optimize

was MS (Murashige T. & Skoog F., 1962) medium supplemented with 5 g/l agar, 100 ml/l coconus water, 5.5 g/l agar. The pH of the medium was adjusted to 5.8 using 0.1N NaOH /0.1N HCl. The culture vessels containing the medium were autoclaved at 121^{o}C at 1.1 atm for 15 minutes. Bulb sildes with 1.5 - 2 mm in size were cultured in medium with several modifications such as saccharose, glucose from 30 to 150 g/l; BA from 0 - 1 mg/l and α-NAA from 0.01 to 0.3 mg/l and kept in darkness or 16 h photoperiod of 20 Klx light intensity lamps and kept at 22 ± 3^{o}C. After 4 week, the bulble forming rate the weight of bulblet and multiplied coefficient were calculated and analyzed. The rooting ability of bulblet was also tested after transferring onto rooting medium MS added α- NAA (0.2 - 1.5 mg/l).

b. Studying effect of some factor on bulblet development in garden:

Different weigh bulblets treated by cold condition in 4, 6, 8 or 10 weeks were planted in garden. After 4 week planting, data of the survival rate, the height, number of leaf were collected and analyzed was used to tested *in vitro* culturing or gardening.

c. Data analysis:

The data was analyzed using the IRRISTAT 5.0 software.

III. RESULTS

3.1. Effect of some factor on reorganizing new lily bulblet

The effects of difference concentrations of saccharose and glucose on the bulblet forming rate were showed in Table 1. These results demonstrated that the bulbscale slide culturing in medium added 60 g/l saccharose and 30 g/l glucose formed the best new bulblet with the bulblet forming rate reached 81.25%, the coefficient equal to 3.13.

Table 1. Effect of sugar on bulblet formation of lily bulb scale slide in vitro

Sugar	Amount (g/l)	Rate of bulblet fornating	Coefficient	Characteristic
Saccharose	30	52.09 ± 1.8^{FGHbc}	1.35 ± 0.05^{GHc}	Small
	60	57.29 ± 1.8^{EFb}	2.06 ± 0.14^{Cb}	Average
	90	77.08 ± 1.8^{Aba}	2.99 ± 0.08^{Aa}	Big
	120	59.38 ± 3.1^{DEFb}	2.09 ± 0.1^{Cb}	Average
	150	43.75 ± 5.4^{Hic}	1.19 ± 0.04^{Hc}	Small
	CV%	5.4	4.6	
	LSD	5.68	0.16	
Glucose	30	45.84 ± 1.8^{GHIcd}	1.21 ± 0.08 ^{Hc}	Small
	60	53.13 ± 3.1^{FGbc}	1.67 ± 0.07 ^{EFb}	Average
	90	66.67 ± 3.6 ^{CDa}	2.22 ± 0.13^{Ca}	Big
	120	56.25 ± 3.12^{Fb}	1.60 ± 0.13^{FGb}	Average
	150	42.71 ± 1.16 ^{Id}	1.16 ± 0.05^{Hc}	Small
	CV%	5.3	6.2	
	LSD	5.08	0.18	
Saccharose (S) + glucose(G)	0 S + 0 G	0	0	No bulblet
	30 S + 30 G	65.63 ± 3.1 ^{CDEbc}	1.76 ± 0.02 ^{DEFd}	Small
	30 S + 60 G	68.72 ± 3.2 ^{BCb}	2.65 ± 0.09^{Bb}	Average
	30 S + 90 G	60.42 ± 1.8 ^{CDEFbc}	1.93 ± 0.09^{CDEcd}	Average
	60 S + 30 G	81.25 ± 3.1 ^{Aa}	3.13 ± 0.09^{Aa}	Big
	60 S + 60 G	58.34 ± 4.8 ^{DEFc}	2.04 ± 0.19^{CDc}	Average
	90 S + 30 G	66.67 ± 1.8 ^{CDbc}	1.67 ± 0.05^{EFd}	Average
	CV%	5.1	5.0	
	LSD	5.08	0.16	

In each column, means followed by the same letters are not significantly different using at 5% probability level a,b,c,d… means the different among the formulas in each type of sugar, ABCD… means the difference between the formulas of all three types of sugar.

Zaghmout and Lorres (1985) said that saccharose or glucose was suitable for bulblet formation. Besides that, they also confirmed that sugar concentration closely related with organization of cultured tissue because of that it stimulated cell to reorganize, provided good vitality to tissues and organs. Pelkonen V. P. (2005) declared that most of species were usually cultured in medium with sugar concentration from 2 - 6% but bulblet formation in medium with 9 - 12% or higher.

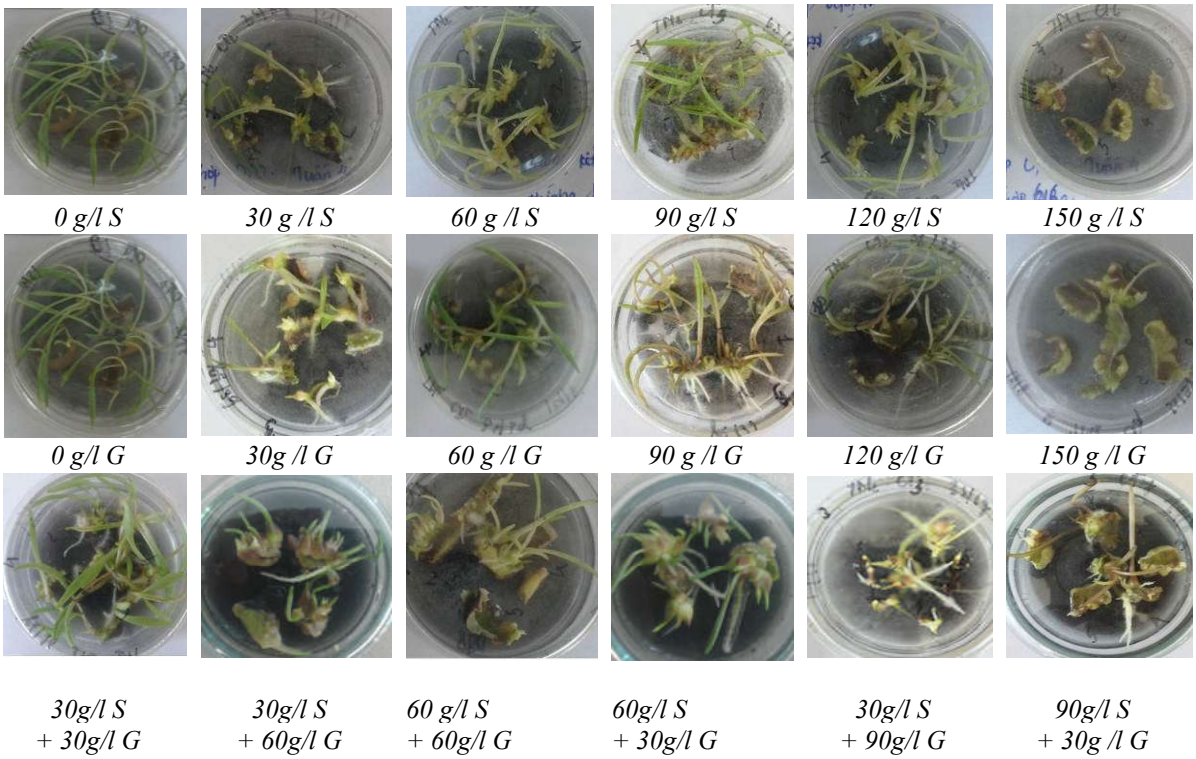


Figure 1. Bulblet formation of lily bulb scale slide in medium added glucose (G) or saccharose (S) with different concentration

Beside culturing medium, the materials with different species, age, location, sample size played important roles in bulblet formation (Duong Tan Nhut et al., 2005). The results I in Table 2 and Fig. 2 showed that, the first type of slide (at lowest) had the highest rate of plant regeneration (84%), the highest multiplying coefficient (3.03) with big and uniform size. This result was consistent with the previous report of Duong Tan Nhut et al. (2006).

Table 2. Ability of bulblet formation from different lily bulb scale slide

Kind of slide	Rate of bulblet forming (%)	Coefficient
Type 3	43.33 ± 3.3^{c}	0.71 ± 0.02^{c}
Type 2	62.22 ± 1.9^{b}	2.15 ± 0.16^{b}
Type 1	84.44 ± 1.9^{a}	3.03 ± 0.07^{a}
CV%	3.9	5.0
LSD	4.5	0.12

In each column, means followed by the same letters are not significantly different using at 5% probability level
Culturing medium: 60 g/l saccharose + 30 g/l glucose + 100 ml/l coconut water + 1 g/l activated charcoal;
Type 1: lowest slide; Type 2: middle slide; Type 3: highest slide

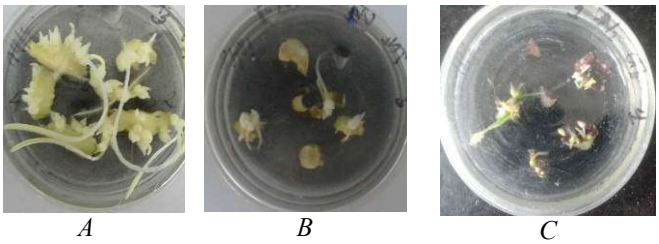


Figure 2. Bulblet formation from lowest slides (A), middle slides (B) and highest slides (C) of bulbscale

Our experiment indicated that BAP strongly affected the bulblet formation. The highest formed new bulblet of 85.56% and the highest coefficient of 3.52 were found in MS medium supplemented with 0.5 mg/l BAP (Table 3). When testing bulblet formation from original bulble slides in MS medium added combination of 0.5 mg/l BAP and different α-NAA concentration, the results in Table 4, Fig 3 showed that the combination of 0.5 mg/l BAP and 0.1 g/l NAA stimulated 84.44% of slides having new bulblet with the coefficient of 3.6. The results strengthen the idea of Takayama & Misawa (1979), in which they indicated the low concentration of auxin NAA and cytokinin BAP to promote the bulblet formation. Phytohormone obviously plays a significantly important role in stimulating the growth, development and differentiation of organs. BA (6-benzylaminopurine) belonging to the cytokinin group required for cell division, enhanced shoot generation (Duong Tan Nhut, 2006).

Table 3. Effect of BA on bulblet formation from lily bulb scale slide

BA (mg/l)	Rate of bulblet formation (%)	Coefficient
0.00	73.33 ± 3.33^{c}	2.8 ± 0.07^{e}
0.05	77.78 ± 1.98^{bc}	2.9 ± 0.07^{de}
0.10	81.11 ± 1.86^{ab}	3.05 ± 0.1^{cd}
0.30	82.22 ± 1.82^{ab}	3.14 ± 0.02^{bc}
0.50	85.56 ± 1.92^{a}	3.52 ± 0.07^{a}
1.00	80 ± 3.33^{abc}	3.27 ± 0.05^{b}
CV %	3.1	2.2
LSD	4.41	0.12

Table 4. Effect of BA and NAA on bulblet formation from lily bulb scale slide

BA (mg/l)	α-NAA (mg/l)	Rate of bulblet formation (%)	Coefficient
0.5	0.01	72.22 ± 5.09^{b}	2.88 ± 0.09^{c}
	0.03	73.33 ± 3.34^{b}	3.07 ± 0.05^{bc}
	0.05	80.00 ± 3.33^{ab}	3.14 ± 0.07^{b}
	0.10	84.44 ± 1.93^{a}	3.60 ± 0.07^{a}
	0.30	73.33 ± 3.33^{b}	2.93 ± 0.09^{c}
CV%		4.6	2.4
LSD		6.46	0.14

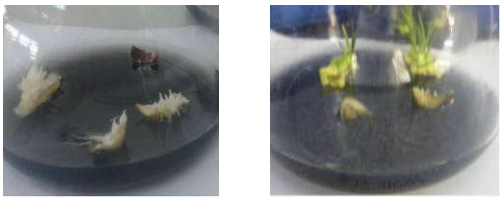
In each column, means followed by the same letters are not significantly different using at 5% probability level
Culturing medium: 60 g/l saccharose + 30 g/l glucose + 100 ml/l coconut water + 1 g/l activated charcoal;

A *B* *C* *D* *E*

Figure 3. Bulblet formation in medium added BAP and α-NAA

A. 0.5 mg/l BA+0.01 mg/l NAA; B. 0.5 mg/l BA+0.03 mg/l NAA
C. 0.5mg/l BA+0.05 mg/l NAA; D. 0.5 mg/l BA+0.1mg/l NAA; E. 0.5 mg/l BA+0.3 mg/l NAA.

Culture condition such as light regime also found to be affected the formation of bulblet formation *in vitro*. However, there were few reports on this issue in Lily tissue culture (Pelkonen, 2005). Our results in Table 5, Fig 4 revealed that the darkness stimulated callus and bulblet formation and the light promoted shoot and leaf production. These findings were similar to research of Maesato *et al.* (1994) và Niimi *et al.* (1997). Light condition was one of the most important physical factors in promoting reorganization (Tisserat 1987, 1990) or cell division (Bach & Swiderski 2000).



A *B*

Figure 4. Bulblet formation from bulbscale slides culturing in dark (A) and 16h dark/8h light (B)

Table 5. Effect of Lighting mode on bulblet formation from Lily bulb scale slide

Lighting mode	Rate of bulblet forming (%)	Coefficient	Characteristic of new bulblet
(*1)	83.33 ± 3.3[a]	3.68 ± 0.05[a]	white, big, no leaves
(*2)	78.89 ± 5.09[a]	3.34 ± 0.1[b]	green, small, having leaves
CV%	5.3	2.2	
LSD	9.7	0.18	

*Note: (*1)24 h in dark; (*2) 16h light/8h dark*

3.2. Effect of some factor on lily bulblet number in vitro

Ilcheva, Stanilon and Zagorska (1993) said that bulble or bulbscale had highest tolerance in bulblet forming *in vitro*. It was similar to declaration of Duong Tan Nhut et al. (2006). The result in Table 6 shows that while MS medium added 0.1 mg/l BA promoted the growth of lily bulblet, MS medium added 0.1 mg/l BA and 0.1 mg/l NAA supported bulblet production in weight with average weight of 0.54 g/bulblet. Although, the weight of bulblet in medium added 0.1 mg/l BA and 0.3 mg/l NAA (Table 7) was the highest but bulblet had also some roots.

Table 6. Effect of BA on growing of lily bulblet in vitro

BA (mg/l)	Initial weight (g)	weight after 4 weeks (g)
0.00	0.14±0.012[a]	0.30±0.02[c]
0.05	0.13±0.013[a]	0.45±0.02[b]
0.10	0.14±0.011[a]	0.54±0.03[a]
0.30	0.12±0.016[a]	0.40±0.02[b]
0.50	0.13±0.013[a]	0.33±0.03[c]
CV%	5.6	5.5
LSD	0.13	0.4

Table 7. Effect of BA and α-NAA on growing of lily bulblet in vitro

BA (mg/l)	α-NAA (mg/l)	Initial weight (g)	weight after 4 weeks (g)
0.1	0.01	0.14±0.013[a]	0.36±0.013[e]
	0.03	0.14±0.010[a]	0.40±0.015[d]
	0.05	0.14±0.015[a]	0.47±0.020[c]
	0.10	0.14±0.011[a]	0.51±0.020[b]
	0.30	0.14±0.013[a]	0.60±0.020[a]
CV%		3.6	3.0
LSD		0.94	0.25

In each column, means followed by the same letters are not significantly different using at 5% probability level
Culturing medium: 60 g/l saccharose + 30 g/l glucose + 100 ml/l coconut water + 1 g/l activated charcoal.

Table 8. Effect of α-NAA on rooting of bulblet Lily in vitro

NAA (mg/l)	Rate of rooting (%)	Lengh (cm)	Number of root	Characterictis
0.2	60.00^{d}	1.80^{d}	1.29^{c}	Small and average roots; big bulblet
0.5	68.89^{c}	2.17^{c}	2.66^{b}	Big, uniform roots; average bulbet
1.0	80.00^{b}	2.76^{a}	3.13^{a}	Average, uniform roots; average bulbet
1.5	97.78^{a}	2.56^{b}	2.98^{a}	Average, uniform roots; average bulbet
CV %	5.1	5.2	4.7	
LSD	6.3	0.16	0.23	

Culturing medium: MS+ 60 g/l saccharose+30 g/l glucose+ 100 ml/l coconut water + 1 g/l activated charcoal.

0.2 mg/l NAA *0.5 mg/l NAA* *1 mg/l NAA* *1.5 mg/l NAA*

Figure 5. The rooting of in vitro bulblet in medium added different concentrations of α-NAA

In order to test the rooting cappacity, bulblet was cultured in MS added α-NAA at different concentration. The result in table 8 shows that 80% bulblet formed root in MS medium added 1 mg/l NAA with average root number and root length of 3.1 root and 2.76 cm, respecdtively. The result was similar with publishcation of Pandey R.K., Singh A.K. and Mamta Sharma (2009). However, in MS medium added 0.2 mg/l α-NAA, although the root indexts were low, the bulblet became bigger than other on other mediums (Fig. 5).

3.3. Development of lily bulblet in green houses

Table 9. Effect of bulblet weight and cold pretreatment time on lily plantlet development in the green house

Time of cold treatment	4 weeks	6 weeks	8 weeks	10 weeks
Weigh of bulblet (g)	The rate of survival (%)			
< 1	70.00	66.67	76.67	73.33
1.5	76.67	76.67	83.33	76.67
2.5	76.67	83.33	83.33	76.67
>3	86.67	93.33	90	93.33
Weigh of bulblet (g)	Height of plantlet (cm)			
< 1	3.69 ± 0.05^{ABc}	3.84 ± 0.06^{Ac}	3.49 ± 0.12^{Bc}	3.75 ± 0.06^{Ac}
1.5	3.95 ± 0.06^{Abc}	3.94 ± 0.06^{Abc}	3.84 ± 0.1^{Abc}	3.87 ± 0.03^{Abc}
2.5	4.06 ± 0.12^{Aab}	4.02 ± 0.03^{Aab}	4.00 ± 0.07^{Aab}	4.28 ± 0.02^{Aab}
>3	4.29 ± 0.15^{Aa}	4.10 ± 0.06^{Aa}	4.17 ± 0.05^{Aa}	4.38 ± 0.05^{Aa}
Weigh of bulblet (g)	Number of leaf (p)			
< 1	2.44 ± 0.1^{Aa}	2.36 ± 0.05^{Aa}	2.43 ± 0.13^{Aa}	2.36 ± 0.06^{Aa}
1.5	2.37 ± 0.07^{Aa}	2.59 ± 0.1^{Aa}	$2.52 \pm 0,1^{Aa}$	2.37 ± 0.11^{Aa}
2.5	2.52 ± 0.14^{Aa}	2.50 ± 0.07^{Aa}	2.64 ± 0.07^{Aa}	2.54 ± 0.1^{Aa}
>3	2.56 ± 0.1^{Aa}	2.57 ± 0.06^{Aa}	2.67 ± 0.05^{Aa}	2.81 ± 0.09^{Aa}

abc is different value of the formula in each column; ABC is different value of the formula in each row; means followed by the same letters are not significantly different using at 5% probability level.

Bulblets weighed from 0.5 to 4 g were cold treated and transferred to green house. The result in Table 9 showed different bulblet weigh and cold pretreatment time affected of the development of lily bulblet in the green houses. Without cold pretreatment, all bulblets were died. In contract, bulblets kept in $4 \pm 1\ ^0$C for chosen periods had the high rate of survival, over 66.67%. However, the different cold pretreatment periods from 4 to 10 weeks did not demonstrate the different effects on survival rate, plant height and number of leaves of bulblets, which found to be influenced by the weight of bulblet. As indicated by Nguyen Thi Ly Anh (2005), the *in vitro* 5^{o}C pretreatment bulblets with the weight higher than 1 g were easily adaptive and developed into strong and health plants in green house. These results also showed that the heavier the bulblet was, the higher of survival rate, the height and number of leaf of the bulblet had.

IV. CONCLUSION

1. MS medium + 60 g/l saccharose + 30 g/l glucose made 81.25% bulbscale creat new bulblet with high coefficient, 3.13. Lowest lily bulbsacle slide had the highest rate of bulblet forming 84.44% with the highest ecofficent; the new bulblets were big and uniform.

2. MS medium + 60 g/l saccharose + 30 g/l glucose + 100 ml/l coconut warter + 5.5 g/l agar +1 g/l activated charcoal + 0.5 mg/l BA + 0.1 mg/l NAA stimulated bulb scale slide forming new bulblet with coefficient was 3.6. Moreover, in the dark condition, the bulblet forming was well with the multiplied ecofficent was 3.68.

3. MS medium + 60 g/l saccharose + 30 g/l glucose + 100 ml/l coconut warter + 5.5 g/l agar +1 g/l activated charcoal + 0.1 mg/l BA and 0.1 mg/l NAA was the best for growing lily with uniform and high quality. MS added 0.2 mg/l NAA was good condition for bulblet growing and rooting.

4. The weigh of bulblet affected to the survival rate, the height and number of leaf of the bulblet.

Acknowledgment

This study was conducted under financial support by ARES with the Belgian Development Cooperation Fund for the project "Improving some stress tolerances in lily plant by genetic engineering".

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TỐI ƯU QUY TRÌNH NHÂN GIỐNG *IN VITRO* NGUỒN GEN LILY QUÝ

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TÓM TẮT

Lily là cây hoa quan trọng, có giá trị kinh tế và hiện đang được sản xuất ở quy mô công nghiệp ở nhiều nước, trong đó có Việt Nam. Tuy nhiên để cung cấp số lượng lớn cây con, việc ứng dụng công nghệ tái sinh và nhân chồi vô tính *in vitro* cần được nghiên cứu cho từng loài. Trong nghiên cứu này, một số yếu tố ảnh hưởng lớn sự tạo củ từ lát cắt vảy củ *in vitro* đã được nghiên cứu và tối ưu. Các lát cắt vảy củ lily *in vitro* gần đĩa gốc được nuôi cấy trên môi trường MS có bổ sung 60 g/l saccharose, 30 g/l glucose, 0,5 mg/l BA, 0,1 mg/l NAA, 100 ml/l nước dừa, 5,5 g/l agar và 1 g/l than hoạt tính trong điều kiện tối hoàn toàn sẽ cho tỷ lệ tái sinh củ cao nhất 3,68 củ/lát cắt vảy củ. Các củ lily *in vitro* sinh trưởng lớn lên về khối lượng khi được nuôi cấy trên môi trường MS bổ sung 0,1 mg/l BA và 0,3 mg/l NAA; ra rễ tốt ở môi trường MS có 0,2 mg/l NAA. Ở giai đoạn ra rễ, củ lily phát triển lớn và ra rễ ở môi trường MS có 0,2 mg/l NAA. Xử lý lạnh là yêu cầu cần thiết cho sự sống sót củ lily ngoài vườn ươm. Tuy nhiên, các củ được xử lý lạnh ở 4, 6, 8 hay 10 tuần không có sự sai khác về tỷ lệ sống hay chiều cao cây và số lá trên cây, mà khối lượng củ càng lớn thì cây có các chỉ số này càng lớn.

Từ khóa: *Lilium* spp, Lily, nhân giống in vitro, nuôi cấy mô.

Received : 03/9/2017
Revised : 28/9/2017
Accepted : 12/10/2017