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Bulblet formation from bulbscale segments of *Lilium* using bioreactor system

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Abstract

In vitro bulblet formation was studied using solid, liquid and bioreactor culture (immersion and periodic immersion in liquid media using ebb and flood) in order to develop a cost effective method for the mass propagation of *Lilium* oriental hybrid 'Casablanca'. Although the percent of bulblet formation was higher in solid culture, the increased growth rate and production of large number of bulblets in bioreactor makes it suitable for mass propagation. Four times per day and 15 min of medium supply was optimal for bulblet formation in ebb and flood bioreactor. Bulblet formation was also found to be effective in 16-h photoperiod. It was also observed that bulblet formation in the medium with 1.0 mg dm^{-3} BA and 0.3 mg dm^{-3} NAA was higher than in the medium without growth regulators, but formation of abnormal bulblets was higher in medium with BA and NAA.

Additional key words: ebb and flood, liquid culture, lily, micropropagation, photoperiod, solid culture.

Introduction

Lilium is one of the important floricultural crops for bulb and cut flower production. Numerous studies have been reported on regeneration of bulblets from excised lily bulbscales (Robb 1957, Hackett 1969, Allen 1974, Anderson 1977, Novak and Petru 1981, Takayama and Misawa 1983, Varshney *et al.* 2000) and it is now the current method for vegetative propagation of lilies. Bulblets have several advantages for mass propagation of *Lilium* because they are easier to handle and can be sown as seed, thereby decreasing the labour cost, they can be transported dry and no extra time or facilities are needed for hardening off, they are hardier, thereby increasing the survival rate after transplantation. But in general, the commercial use of micropropagation is currently being reduced because of high production cost resulting primarily from high labour costs.

Much research has been conducted recently on bioreactor in micropropagation because it offers various advantages over conventional micropropagation procedures due to possibilities of automation, saving labour cost and scale up for mass propagation (Paek *et al.* 2001). Accordingly, cells, somatic embryos or organogenic propagules like bulblets, corms, microtubers or shoots have been cultured in liquid suspension in bioreactors (Stuart *et al.* 1987, Akita and Takayama 1988, Preil and Beck 1991, Takahashi *et al.* 1992, Cantliffe *et al.* 1993, Gupta *et al.* 1993, Akita *et al.* 1994, Seon *et al.* 2000). However, upto now only a few reports on *Lilium* propagation in bioreactor have been published. From these paper it is difficult to ascertain the progress achieved in *Lilium*. Takayama *et al.* (1986) reported on bioreactor experiments with *Lilium* using tissue with multiple buds as inoculum. Similarly Seon *et al.* (2000) and Lim *et al.* (1998) reported the effect of bioreactor types on bulblet growth. So far our knowledge no reports have been found on bulblet formation from bulbscale segments using bioreactor culture.

In this paper we examine factors affecting the formation of bulblets *in vitro* using solid, liquid and balloon type bubble bioreactor (immersion and ebb and flood) in order to develop a cost effective method for commercial micropropagation.

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Abbreviations: BA - N^6-benzyladenine; MS - Murashige and Skoog; NAA - 1-naphthaleneacetic acid.

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Materials and methods

Plants: The bulblets of *Lilium* oriental hybrid 'Casablanca' (derived in Holland 1984 from crosses between *Lilium speciosum*, *L. auratum*, *L. rubellum* and *L. nobillissimum*) were maintained on Murashige and Skoog's (1962, MS) medium supplemented with 9 % sucrose and 0.24 % gelrite (*Duchefa*, Haarlem, The Netherlands). When the bulblets grown to 2 g, 1 - 2 peripheral scales were removed and middle scales were excised and were then cut into 1 mm^2 size sections and used as a source of explants for this experiment.

Solid culture: Scale segments (1 g) were inoculated to cylinder vessels containing MS medium supplemented with 0.3 mg dm^{-3} 1-naphthaleneacetic acid (NAA), 1.0 mg dm^{-3} N^6-benzyladenine (BA), 3 % sucrose and 0.24 % gelrite. The pH of the medium were adjusted to 5.8 before autoclaving at 121 °C and 1.2 kg cm^{-2} pressure for 15 min.

Liquid culture: Bulbscale segments (11 g) were cultures in Ehrlenmeyer flasks (400 cm^3) each containing 200 cm^3 liquid medium (same as solid culture except gelrite). The flasks were agitated on a horizontal gyrotory shaker at 100 rpm.

Bioreactor culture: Two types of culture system, immersion and ebb and flood (Fig. 1), were used to select a suitable method for bulblet formation. Scale segments (17 g) were transferred to 5000 cm^3 balloon type bubble bioreactor (BTBB) with 1 dm^3 MS liquid medium supplemented with 3 % sucrose, 0.3 mg dm^{-3} NAA and 1.0 mg dm^{-3} BA. The pH of the medium were adjusted to 5.8 before autoclaving at 121 °C and 1.2 kg cm^{-2} pressure for 40 min. The volume of input air was adjusted to exchange 0.1 volume of culture air per min. In case of immersion type, bulblets were submerged in liquid during the whole period but in case of ebb and flood, system was programmed to immerse the bulblets in medium for 0.30 h and dry for 5.70 h by timer and solenoid valve. All cultures were maintained at 25 ± 2 °C temperature, 16-h photoperiod (irradiance of 40 μmol m^{-2} s^{-1}) for the period of 4 weeks.

In order to develop a suitable bioreactor system (ebb and flood), we examined different factors like number (the number of medium supply was 4, 6 and 8 times per day for 30 min every time) and duration of medium supply (medium was supplied 4 times per day for 15, 30, 60 or 120 min) on bulblet formation during bioreactor culture. We also examined the bulblet formation on growth regulator free medium under complete darkness or 16-h photoperiod.

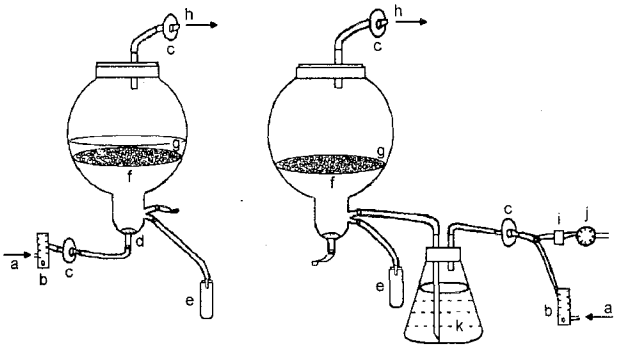

Fig. 1. Schematic diagram of two different type of bioreactor system: a - air inlet, b - air flow meter, c - membrane filter, d - glass sparger, e - sampling port, f - supporter (net), g - bulblet scale segments, h - air outlet, i - timer, j - solenoid valve, k - medium reservoir.

Experimental design and data analysis: All experiments were repeated three times with 3 replicates. The percentage of bulblet formation, number of bulblets per vessels and the fresh mass of segments was investigated after 4 weeks of culture. Data were subjected to Duncan's multiple range test using *SAS* program (*Version 6.12*, SAS Institute Inc., Cary, USA).

Results and discussions

Comparative studies between liquid, solid and bioreactor culture (immersion and periodic immersion in liquid media using ebb and flood) revealed that bulblet formation was most efficient in solid culture (Table 1, Fig. 2). No bulblet formation was observed in liquid and immersion type bioreactor culture. It indicates that complete immersion of bulbscale into the liquid media inhibit the bulblet formation. Ebb and flood bioreactor system functioning on the principle of temporary immersion enabled a constant supply of nutrients as well as aeration to explants leading to the bulblet formation. Although the percentage of bulblet formation was lower in ebb and flood system (51.7 %), but we have harvested over 1000 bulblets from each batch of culture (Table 1). Thus bulblets culture in bioreactor (ebb and flood) will reduce the labour manipulations required for solid culture and facilitates scaling up of bulblet production.

In order to increase the rate of bulblet formation, we examined different factors like number and duration of medium supply, effect of light and dark condition on bulblet formation in bioreactor. The highest percentage of bulblet formation (75.8 %) was observed when medium

was supplied 4 times per day for 15 min and the efficiency of bulblet formation was almost similar to the solid culture (Tables 2, 3). Higher than 15 min medium supply decreased the percentage of bulblet formation as well as number of bulblets per vessel during bioreactor culture. Immersion of bulbscale into liquid media for longer time inhibit the bulblet formation. This may relate to the surface contact of bulbscale with the air, longer duration of surface contact with air stimulate the bulblet formation from bulbscale during bioreactor culture.

Table 1. Effects of culture method on bulblet formation in scale segment culture of *Lilium* oriental hybrid 'Casablanca' after 4 weeks of culture. The value of growth rate is the quotient of the fresh mass after 4 weeks and the fresh mass of the inoculum. Means with diferent letters within columns are significantly different according to Duncan's multiple range test at $P \leq 0.05$.

Culture method	Bulblet formation [%]	Number of bulblets [vessel^{-1}]	Segment fresh mass [g]	Growth [folds]
Suspension	0	0	32.8b	3.0b
Immersion	0	0	46.4b	2.9b
Ebb and flood	51.7b	1025.5a	70.9a	4.1a
Solid	78.7a	81.9b	14.0c	3.4ab

Table 2. Effects of the number of medium supply per day in ebb and flood type bioreactor culture on bulblet formation in scale segment culture of *Lilium* after 4 weeks of culture. Medium was supplied for 30 min per time. Means with diferent letters within columns are significantly different according to Duncan's multiple range test at $P \leq 0.05$.

Medium supply [number d^{-1}]	Bulblet formation [%]	Number of bulblets [vessel^{-1}]	Segment fresh mass [g]	Growth [folds]
4	51.7a	1025.5a	70.9a	4.1a
6	19.3b	369.6b	36.0b	2.1b
8	7.2c	139.5c	32.8b	1.9b

The percent of bulblet formation was much higher in media containing 0.3 mg dm^{-3} NAA and 1.0 mg dm^{-3} BA as compared with media without growth regulators (Table 4, Fig. 3*A*,*C*). When the bulblets formed in media containing 0.3 mg dm^{-3} NAA and 1.0 mg dm^{-3} BA were transferred in media with 9 % sucrose without growth regulators, numerous abnormal bulblets were observed after 8 weeks of culture (Fig. 3*B*). The bulblets which were initiated on growth regulator free medium, showed no sign of abnormality even after 16 weeks of bulbing (Fig. 3*D*). Addition of growth regulators sometimes may bring about morphological abnormalities and hyperhydricity as has been reported in several plant species (Bhojwani and Razdan 1996). Since the abnormal bulblets mean a lower survival rate during transplantation, we recommended using medium without growth regulator for *Lilium in vitro* bulblet formation using bioreactor (one step culture system including two stage: bulblet formation and bulbing).

Table 3. Effects of duration of medium supply on bulblet formation in scale segment culture of *Lilium* after 4 weeks of culture in ebb and flood type bioreactor. Medium was supplied 4 times per day. Means with diferent letters within columns are significantly different according to Duncan's multiple range test at $P \leq 0.05$.

Duration of medium supply [min]	Bulblet formation [%]	Number of bulblets [vessel^{-1}]	Segment fresh mass [g]	Growth [folds]
15	75.8a	1451.7a	82.5a	4.9a
30	51.7b	1025.5b	70.9a	4.1a
60	10.8c	197.0c	46.7b	2.9b
120	2.3d	32.1d	47.0b	2.8b

Table 4. Bulblet formation in scale segment culture of *Lilium* oriental hybrid 'Casablanca' in ebb and flood bioreactor as affected by 0.3 mg dm^{-3} NAA and 1.0 mg dm^{-3} BA in the medium after 4 weeks of culture. Medium was supplied 4 times per day for 15 min. Means with diferent letters within columns are significantly different according to Duncan's multiple range test at $P \leq 0.05$.

Growth regulators	Bulblet formation [%]	Number of bulblets [vessel^{-1}]	Segment fresh mass [g]	Growth [folds]
0	61.5b	413.3b	36.8b	2.3b
NAA+BA	75.8a	1451.7a	82.5a	4.9a

Table 5. Effects of light (16-photoperiod) and continuous dark on bulblet formation in scale segment culture of *Lilium* after 4 weeks in ebb and flood bioreactor. Medium was supplied 4 times per day for 15 min. Means with different letters within columns are significantly different according to Duncan's multiple range test at $P \leq 0.05$.

Culture method	Bulblet formation [%]	Number of bulblets [vessel^{-1}]	Segment fresh mass [g]	Growth [folds]
Light	61.5a	413.3a	36.8a	2.3a
Dark	50.2b	360.5b	34.2a	2.0a

In our experiment there was an increase in bulblet formation percentage under a 16-h photoperiod compared to 24-h darkness, but the number of bulblets formed per explant was not influenced (Table 5). Similar response

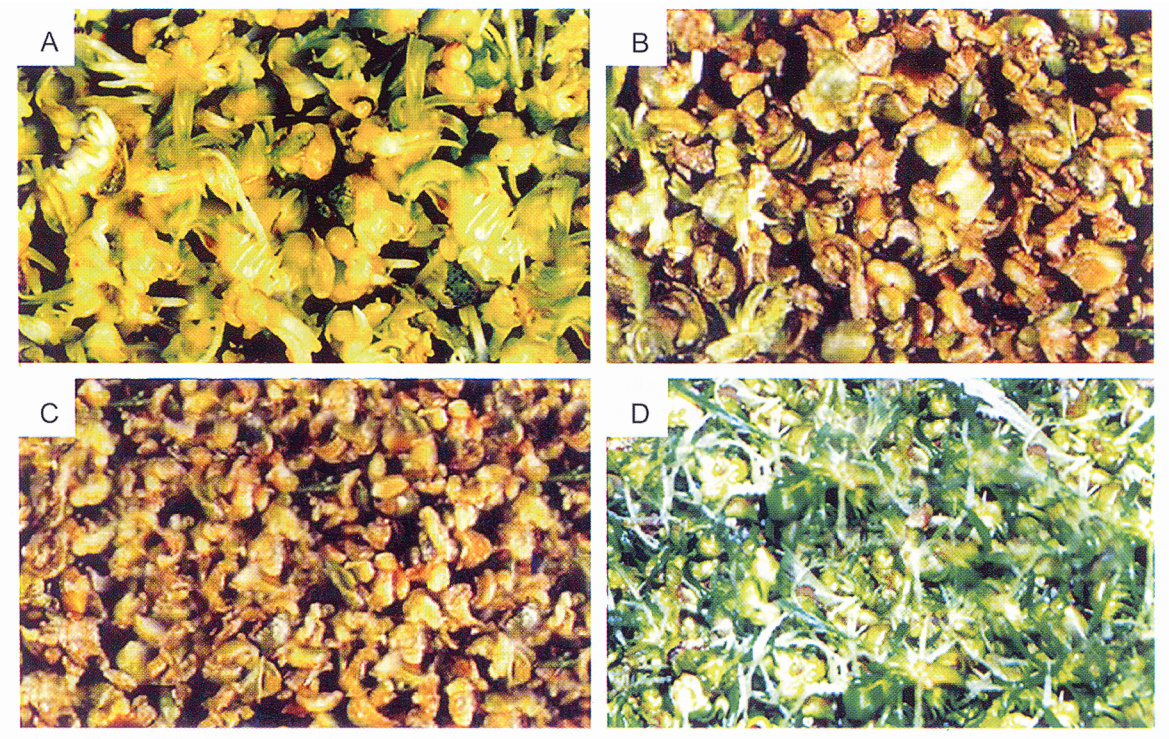

Fig. 2. Bulblet formation in *Lilium* oriental hybrid 'Casablanca': *A* - solid culture, *B* - liquid culture, *C* - immersion culture in bioreactor, *D* - ebb and flood culture in bioreactor (medium supply 4 times per day for 15 min).

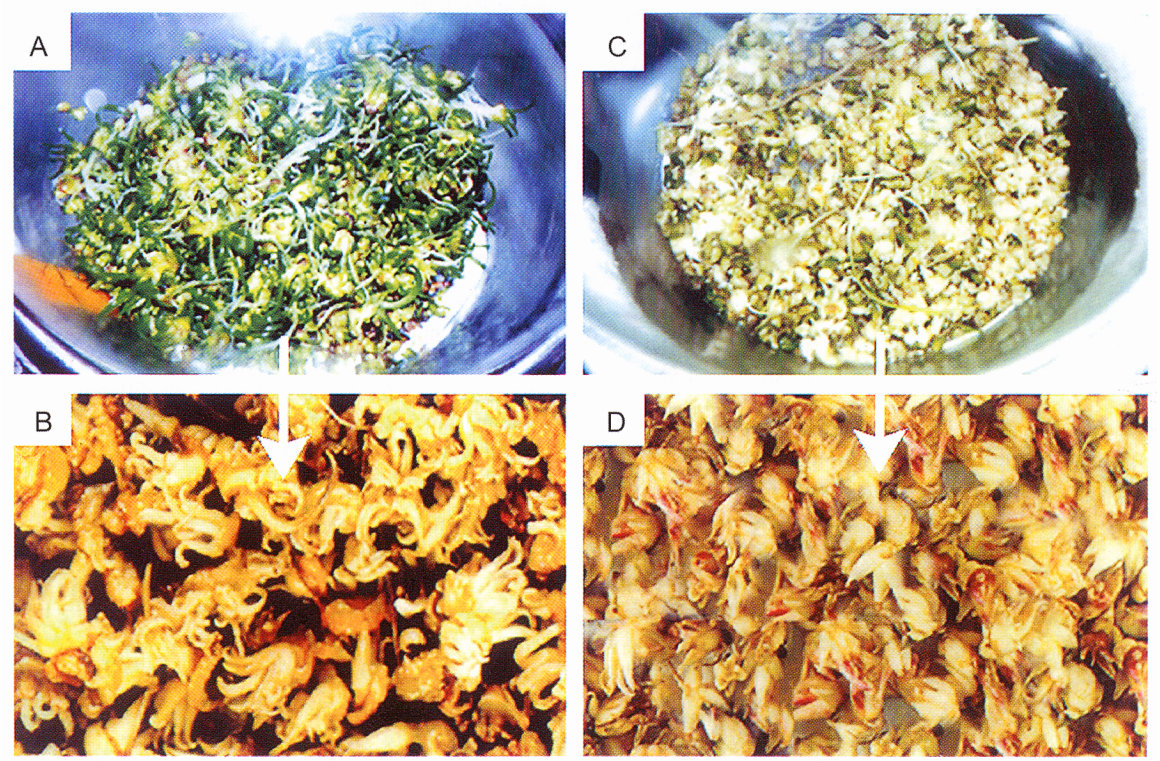

Fig. 3. Bulblet formation and bulbing of *Lilium* oriental hybrid 'Casablanca'. *A* - bulblet formation (3 % sucrose with 0.3 mg dm^{-3} NAA and 1.0 mg dm^{-3} BA for 4 weeks), *B* - bulbing (9 % sucrose for 8 weeks), *C* - bulblet formation (3 % sucrose without NAA and BA for 4 weeks), *D* - bulbing (9 % sucrose for 16 weeks).

was observed by Varshney *et al.* (2000) during *in vitro* mass propagation of *Lilium* Asiatic Hybrid. Maesato *et al.* (1994) found that continuous illumination during culture stimulated bulblet production of *Lilium japonicum*. Contradictory to this, continuous darkness during culture increased bulblet size and number during micropropagation of *Lilium longiflorum* (Stimart and Ascher 1978), whereas Leshem *et al.* (1982) found no difference in the effect of dark and light on percentage regeneration for *Lilium longiflorum*. Such variation in regeneration potential due to physiological status of the material (cultivar, age, time of culture, *etc.*), as has been reported by many researchers working on bulbous plants (Stimart and Ascher 1978, Leshem *et al.* 1982, Niimi 1985, Maesato *et al.* 1994).

Propagation of *Lilium* through tissue culture has already been studied by several groups (Robb 1957, Hackett 1969, Allen 1974, Anderson 1977, Novak and Petru 1981, Takayama and Misawa 1983, Varshney *et al.* 2000). In practice, however, these technique requires a large number of vessels as well as considerable labour to produce numerous bulblets. This method presented in this study is efficient and cost effective for the mass propagation of *Lilium* using bioreactor.

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