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In vitro propagation of lily (*Lilium longiflorum* T.) cv. Pavia in continuous immersion bioreactor (CIB)

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Abstract
In the global flower industry, lily (*Lilium longiflorum* T.) holds 4th position among top 10 cut flowers and 2nd among geophytes. Conventional micropropagation technique using solid media is a typically labor-intensive method of producing elite clones of lily cultivars. Commercially, micropropagation is more important than seed or vegetative propagation. The present investigation was conducted to assess the viability of Continuous immersion bioreactor (CIB) for development of lily bulbs *in vitro*. To establish the protocol, we used *in vitro* regenerated bulblets of *Lilium* cv. Pavia as starting material. Factors such as additional sucrose, duration of subculture and ventilation were tested. Total fresh weight and bulb size was significantly increased by media refreshment. The sucrose supplementation in MS media also plays a crucial role in growth and development of lilium bulbs. Larger bulblets were formed in MS liquid media supplemented with 9% sucrose. The ideal bulb size (>32.83 mm in circumferences) and weight (>23.76 g) was achieved when bulblets were cultured in CIB for 12 weeks with 4 times media renewal at 2 weeks interval. More than 90% of plantlets survived in a cocopeat and soil (1:1) substrate. This protocol allows the production of lily bulbs in a simple CIB, with effective rooting and acclimatization. This is the first CIB mediated protocol for lilium bulb production in India.

Keywords: Bioreactor, lilium, bulbs, liquid culture, acclimatization

1. Introduction
Lily, a monocotyledonous species belonging to the Liliaceae, is one of the most important cut-flower species. Lilies are economically important mainly because of their large and attractive flowers and very popular in the USA, some European and Asian countries. Lilies are usually propagated by scaling, a technique which produces 3–5 bulbs from each bulb scale, depending on species, cultivar, and scale size. Several authors have shown the possibility to micropropagate the Lilium species through tissue culture in agar-based systems with high coefficients of multiplication and variable subculture times (Kumar *et al*., 2007) [7]. Scale propagation makes it difficult to obtain large numbers of bulbs from disease-free stock or new cultivars in a short period of time (Stimart and Ascher, 1978) [14]. Therefore, micropropagation *in vitro* is essential to produce large numbers of bulblets in lily. In general, Lilium species are still proliferated *in vitro* by a scale-bulblet cycling propagation method. But the investment of facilities, machinery and manual labor in tissue culture have resulted in increased cost. The major cost in plant propagation by tissue culture is labor. This is especially concentrated in the rooting stage when individual shoots are separated. In order to save labor in tissue culture propagation, Maene and Debergh (1985) [10] added liquid media to proliferating shoots, instead of transferring the tissues to a fresh medium. Recently, it has been proposed the use of liquid medium in bioreactors as possible way of reducing costs of propagation (Adelberg and Fari, 2010) [1]. Bioreactors are vessels designed for large-scale cell, tissue or organ culture in liquid media. Functionally, plant culture bioreactors can be divided into two broad types: those in which the cultures are immersed partially or temporarily in the medium, and those in which the cultures are continuously submerged. Bioreactors provide more precise control of the plant growth gaseous exchange, illumination, medium agitation, temperature and pH than the conventional culture vessels (Ziv, 2000) [16]. Bioreactor based propagation of plants can increase rate of multiplication and growth of cultures and reduce space, energy and labour requirements in commercial micropropagation (Kodym and Zapata, 2001) [6]. Interesting results have been obtained with Lilium shoots incubated in balloon type bubble bioreactors (BTBB) (Paek *et al*., 2001) [3]: multiplication and growth rate results increased, mainly due to better nutrient and water availability in the liquid phase (Lian *et al*., 2003) [8, 9].

With the same BTBB system, Nesi *et al*. (2014) [11] compared the continuous immersion with the temporary immersion in liquid medium (bioreactors with ebb and flood system) and showed that morphological traits and biomass accumulation were more efficient when culturing was performed under continuous immersion. The positive effects of liquid supplements on the promotion of elongation and rooting of micropropagated shoots have been demonstrated (DeRiek *et al*., 1977) [2]. This paper describes a simple method for the micropropagation of *L. longiflorum* 'Pavia' by the addition of supplemented liquid medium to proliferating cultures in continuous immersion bioreactor (CIB).

2. Materials and Methods

2.1 Preparation and maintenance of explants

The present investigation was carried out at Division of Floriculture and Medicinal Crops, ICAR- Indian Institute of Horticultural Research, Hessaraghatta, Bangalore, India (560089). *In vitro* raised bulblets of Lilium cv. 'Pavia' were used as a source of explants for this experiment. The bulblets were subcultured (before shifting into liquid media) with 4-week intervals and maintained at room temperature of 25°C and 16:8 h photoperiod.

2.2 Bioreactor culture

The *in vitro* propagated bulblets were transferred in 5 L stirred jar type continuous immersion bioreactor (CIB). Each bioreactor contained 2 L of MS liquid medium supplemented with different concentration (0%, 3%, 6%, 9% and 12%) of sucrose to determine their effect on bulblet growth under bioreactor culture. The pH of medium was adjusted to 5.8 before autoclaving. For aeration, the volume of input air was adjusted to 0.1 vvm (air volume/culture volume per minute). Bulblets were submerged in liquid during the whole culture period. Cultures were maintained for 8 weeks at dark and a 25°C air temperature. Bulblet weight, increase in bulblet size, and bulblet number per scale were investigated after 8 weeks of culture.

3. Results and Discussion

In the present study, lily bulblets were exposed to different concentration of sucrose. Most of the bulblets formed were small in size when treated with lower concentration of sucrose, however the size of bulblets were increased as the concentration of sucrose was raised in the MS supplemented media. Larger bulblets were formed in MS liquid media supplemented with 9% sucrose. The ideal bulb size (>32.83 mm in circumferences) and weight (>23.76 g) was achieved when bulblets were cultured in CIB for 12 weeks with 4 times media renewal at 2 weeks interval (Table 1). The bulb size was decreased as the concentration of sucrose was increased above the 9% as it caused the necrosis of tissues resulting death of bullets. Sucrose is a suitable source of carbohydrate that is easily assimilated by the bulblets and converted to starch in developing bulblets as reported by Khuri and Moorby, 1995 [5]. Bulblets of Lilium Oriental Hybrid 'Casablanca' grew at a faster rate when the medium was suplimented with 9% sucrose in a Balloon Type Bioreactor culture (Lian *et al*., 2003) [8, 9]. The similar trends were found for the improvement of other character of bulblets. The maximum number of scaly leaves (6.51), maximum number of roots (8.73) and maximum root length (11.51 cm) per bulbs was observed when bulblets were cultured in MS liquid media supplemented with 9% sucrose for 12 weeks (Table 1). Similar results were reported by Yu *et al*. (2000) [15] for potato microtuber growth during bioreactor culture.

Total fresh as well as dry weight of scaly leaf, roots and bulblets of lily was increased markedly with culture medium refreshment within 3 week and compared with those no refreshment (Table 2). The distribution of bulb size also indicates that there is a strong effect of medium renewal on individual bulblet growth inside bioreactor culture. The maximum; fresh weight of scale leaf (183.20 mg), root fresh weight (76.23 mg) and bulb fresh weight (23.76g) was observed with culture medium refreshment within 3 week and minimum with those no refreshment. The shoot relative water content percentage (93.40%) was also found maximum in 9% sucrose with culture medium refreshment within 3 weeks. Hahn and Paek (2005) [3] found that liquid medium produced higher chrysanthemum shoot FW than a gelled medium, but that immersion resulted in significantly lower physiological parameters than an ebb-and-flow bioreactor. Sreedhar *et al*. (2008) [13] found that a bubble column bioreactor produced *Stevia rebaudiana* shoots with an almost ninefold increase in biomass and significantly higher growth parameters than shake culture. Similar results were also reported by Han *et al*., 2004 [4] as better shoot formation in *Lilium longiflorum* 'Georgia' as influenced by addition of liquid medium.

4. Acclimatization of bulbs

Formed bulblets treated with cold temperature at 4°C for 2 months, and after having them washed in running tap water, were transferred to rectangular containers (35cm × 50cm × 10cm) containing cultivation soil mixed cocopeat, and grown in greenhouse. After 2 months, the growth of bulblets was investigated.

Table 1: Effect of additional sucrose on morphological parameters in Lilium bulbs after 12 weeks of culture in Continuous Immersion Bioreactor

Sucrose percentage (%)	Bulb weight (g)	Bulb diameter (mm)	Sale leaves per bulbs	Root length (cm)	Number of roots
0	5.63	15.64	2.25	3.62	4.53
3	6.35	19.50	3.64	5.23	5.12
6	15.62	24.83	4.25	8.41	6.35
9	23.76	32.83	6.51	11.54	8.73
12	16.52	26.31	4.95	9.56	6.92

*Values in parenthesis indicates Arc sin transformation

** Different letter within the columns indicates significant difference between treatments according to Duncan's multiple range test ($P \leq 0.01$)

Table 2: Effect of subculture frequency on physiological parameters of lilium bulb in Continuous Immersion Bioreactor

Subculture frequency	Fresh weight of scale leaf (mg)	Root fresh weight (mg)	Bulb fresh weight (g)	Bulb dry weight (mg)	Shoot RWC (%)
0 week	60.23	16.52	5.63	0.452	87.67
1 week	76.51	24.54	6.35	0.694	89.07
2 weeks	143.53	56.30	15.62	1.352	91.34
3 weeks	183.20	76.23	23.76	1.567	93.40
4 weeks	164.80	88.63	16.52	2.135	87.07

DW dry weight; FW fresh weight; RWC relative water content

*Values in parenthesis indicates Arc sin transformation

** Different letter within the columns indicates significant difference between treatments according to Duncan's multiple range test ($P \leq 0.01$)

5. Conclusions and limitations of the study

A simple and effective protocol for the *in vitro* propagation of *Lilium* using a CIB was developed. The formed bulblets were grown normally and induced flower stems. This method may be very simple and convenient for rapid multiplication of Lilium species. The study only employed a single genotype. To assess the genotype-independent effectiveness of the protocol, or to assess which factors need to be tweaked for individual genotypes, a large-scale experiment is required. In such an experiment, it would be important to also compare the effectiveness of each stage of the micropropagation protocol (shoot induction, shoot development, root induction, acclimatization) with a conventional solid medium-based protocol for each genotype.

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