**Effects of liquid, temporary immersion bioreactor and solid culture systems on micropropagation of *Lilium ledebourii* *via* bulblet microscales — An endangered valuable plant with ornamental potential**

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**Abstract**

*Lilium ledebourii* (Baker) Boiss. (Liliaceae) is a critically endangered lily species native to northern Iran, where it is protected by law. In order to develop a cost effective method for large-scale propagation, the effects of three culture systems (solid, liquid and temporary immersion) and two types of cytokinins viz. BA and TDZ on the *in vitro* plant regenerationof *L. ledebourii* were studied. To establish the protocol, we used *in vitro* regenerated bulblets obtained from bulb scale segments that were cultured on solid Murashige and Skoog (MS) media as starting material. The bulblet microscale transverse thin cell layers were cultured on MS solid medium containing 3% sucrose and different combinations of plant growth regulators. Choice of both, the culture system and the type of cytokinin, affected the differentiation of explants. Two types of calli formed on explants: type I callus was embryogenic, while type II callus was shoot organogenesis. The highest percentages (94%) of embryogenic callus were obtained when calli were transferred on MS solid media supplemented with 0.54 μM α-Naphthaleneacetic acid (NAA) and 0.44 μM BA. In addition, it was also observed that the use of temporary immersion bioreactor resulted in a significantly lower amount of shoot organogenesis than solid culture systems. Seventy percent of the plantlets were successfully acclimatized to *ex vitro* conditions and were phenotypically similar to the mother plants.

**Key words:** Bioreactor, embryogenic callus, *Lilium ledebourii*,liquid culture, organogenesis

**INTRODUCTION**

The monocotyledonous plant *Lilium ledebourii* (Baker) Boiss., commonly known as “Susan-e- chelcheragh” in Persian, is an endangered and the rarest lily, belonging to the family Liliaceae. It is a bulbous perennial herb, a special local plant growing only in the mountains about 1,800 m above sea level in Gilan provinces of northern Iran, having the ability to resist harsh climates with severe cold (Ghahreman 1997, Rechinger 1989, Wendelbo 1977). The plant whole height is 50-150 cm that begin to blossom around the middle of July in the seventh year of its growth period. Leaves are erect, narrow, alternate, lanceolate, 10-14cm×1-2 cm, sessile, flashy. Having a large brilliant white blooms and fragrant, *L. ledebourii* is among the most gorgeous lilies. The species has an excellent vase life, vigorous growth, tolerance to low light and low temperature, and have therefore great interest as ornamental plants and, especially, as cut flowers (Bakhshaie et al. 2010a,b, Ghahreman 1997).

The naturally low rate propagation by seed and rapid eradication of *L. ledebourii* (Azadi and Khosh-Khui 2007, Bakhshaie et al. 2010a,b), is a serious problem hindering the elite reproductive material. Tissue culture systems, and particularly those based on the process of somatic embryogenesis, can considerably increase the propagation rate. Another possibility for increasing the production scale is the use of liquid and bioreactor cultures. As compared to conventional tissue culture techniques using solid medium, liquid cultures combined with mechanization require fewer culture vessels, less labor, utilities and space (Paek et al. 2001, 2005, Takayama and Akita 2005), and as the automation of production requires, among others, the synchronization of embryo development, somatic embryogenesis in liquid culture systems can be a basis for mechanization of *L. ledebourii* bulb production with the use of bioreactors.

Previous works on *Lilium* genus showed that the physical state of medium (liquid or solid) plays a positive role on micropropagation of lilies. In liquid media the initial induction was achieved efficiently while the development and proliferation of somatic embryos of *L. longiflorum* were more rapid in solid medium. Their somatic embryogenesis in liquid culture was highly satisfactory, and the size of the explants affected the proliferation of embryogenic calli in liquid media (Nhut et al. 2006). More efficient procedures for plant regeneration from long-term maintenance of cell suspension cultures of *L. formosanum* (Nakano et al. 2000), cell suspension culture of *L. longiflorum* (Tribulato et al. 1997) and suspension culture-derived protoplasts of *L. formolongi* (Godo et al. 1996, Mii et al. 1994) were reported as well. In addition, numerous studies reported on *in vitro* culture systems of *Lilium* using different explants, but most work has focused on the *Lilium* × *formolongi*, Oriental hybrid, *L. longiflorum* and Asiatic lilies (Godo et al. 1998, Ho et al. 2006, Lian et al. 2003a,b, Paek et al. 2001, 2005, Takayama and Akita 2005, Thakur et al. 2006).

Reports on the role of cytokinins on the micropropagation of *Lilium* species and cultivars are contradictory. Some suggest that cytokinins have a stimulating effect on shoot formation, as observed in Asiatic lilies, Oriental hybrid, *L. ledebourii*, *L. longiflorum*, *L. davidii* and *L. nepalense* (Bacchetta et al. 2003, Bakhshaie et al. 2010b, Han et al. 2004, 2005, LingFei et al. 2009, Nhut et al. 2001a,b, Wawrosch et al. 2001) while in other studies on *L. ledebourii*, *Lilium* × *formolongi* and *L. longiflorum* (Bakhshaie et al. 2010a,b, Ho et al. 2006, Nhut et al. 2001b, 2002, 2006), cytokinins were shown to stimulate somatic embryogenesis and plant regeneration.

Up till now no protocols of *L. ledebourii* reproduction in liquid and temporary immersion bioreactor systems have been elaborated (Azadi and Khosh-Khui 2007, Bakhshaie et al. 2010a,b). In the presented research work, for the first time induction of calli in *L. ledebourii* on the basal Murashige and Skoog (1962) (MS) solid medium and the *in vitro* conversion of calli into plantlets and adventitious organs (shoots) in temporary immersion bioreactor, liquid and solid MS media were tested on bulblet microscale transverse thin cell layer explants. An additional objective of the study was to investigate propagation rates in temporary immersion system, liquid and solid medium for large-scale propagation of uniform plants in order to rescue *L. ledebourii* and to maintain germplasm which may be helpful in domestication of the species.

**Materials and methods**

*Plant material and general culture conditions*

All experiments were performed with *in vitro* bulblet microscale transverse thin cell layers of *L. ledebourii*. They had been induced from bulb scale explants of bulbs grown in a natural forest in the eastern slopes of Alburz Mountains, Gilan, Iran. After surface sterilization the scale explants were incubated in solid MS medium containing 3% (w/v) sucrose, 0.54 NAA and 0.44 μM 6-benzyladenine (BA) for 3 months in darkness at 25 ± 1 ˚C. Afterwards, bulblets were isolated from the scales and bulblet microscale transverse thin cell layers were excised and used as explants for callus inductionas reported by Bakhshaie et al. (2010a).

The solid media were supplemented with 0.8% (w/v) agar and the pH was adjusted to 5.7 using 0.1N NaOH or HCl. All culture media were sterilized by autoclaving at 121 ˚C under a pressure of 120 kPa for 15 min. The cultures vessels were incubated at 25 ± 1 ˚C under a 16 h photoperiod (40 µmol m-2 s-1) of cool white fluorescent lamps.

*Induction of callus*

For callus induction, the bulblet microscale transverse thin cell layers were placed on full-strength solid MS medium containing 3% sucrose and combinations of 0.54 μM NAA and two cytokinins [0.44 μM BA and 0.45 μM thidiazuron (TDZ)] for 90 d at 25 ± 1 ˚C in the dark. Calli were transferred to fresh medium of the same composition every month for further proliferation.

*Solid culture*

For regeneration, the calli (about 400 mg, Fig. 1A, B) were transferred to MS basal medium containing 0.8% agar and 3% sucrose but without plant growth regulators. About 40 ml of medium were dispensed in baby food jars (9 cm height and 7.5 cm diameter). All cultures were incubated at 25 ± 1 ˚C under 16 h photoperiods of cool fluorescent light.

*Liquid culture*

Calli (about 400 mg) were cultured in 250 ml Erlenmeyer flasks containing 40 ml liquid medium (same as solid culture without agar) for plant regeneration. Afterwards, these flasks were closed with two layer of aluminum foil and cultured in the static condition in the light.

*Bioreactor culture*

For plant regeneration, the calli (about 400 mg) were placed in each temporary immersion bioreactor container (RITA®, CIRAD, France) together with 150 ml hormone-free MS liquid medium containing 3% sucrose and incubated at 25 ± 1 ˚C under 16 h photoperiods. The operations and characteristics of the RITA® vessel have been described by Sankar-Thomas et al. (2008). The immersion frequency in the temporary immersion system was 5 min every 6 h.

*Ex vitro transfer*

The regenerated plantlets with well-developed roots were separated from the callus clumps. Then they were transferred individually to moistened, sterile peat:perlite (1:1, v/v) substrate in plastic pots (8 × 7 cm) and placed in a growth chamber at 25 ± 1 ˚C for hardening. The plantlets were completely covered with plastic bags to maintain humidity for one month. During this period of time the polythene bags were gradually perforated to adapt the plants to ambient environmental conditions. Finally, the bags were removed and the plants were maintained in the greenhouse and watered with tap water.

*Data collection and statistical analysis*

The experiment was conducted in an unbalanced completely randomized design repeated at least three times. Data were recorded for two characters after 45 days of culture: survival percentage of calli (explants that regenerated, but not died) and *in vitro* morphogenesis. Statistically significant differences between means were determined using Duncan Multiple Range Test (DMRT) at *P* ≤ 0.05.

**Results and discussion**

*Induction of callus*

Callus induction began within 4-5 weeks of culture with enlargement of the cut edges of the cultured bulblet microscale transverse thin cell layers. Two types of callus developed from the explants: type I callus was embryogenic and had nodular appearance (Fig. 1A), while type II callus was shoot organogenesis and had spongy aspect (Fig. 1B). The embryogenic callus was initiated from explants on MS solid media supplemented with NAA (0.54 μM) plus BA (0.44 μM) and non-embryogenic callus was obtained on a medium containing 0.54 μM NAA and 0.45 μM TDZ (Table 1).

The development of an efficient and reproducible micropropagation protocol is the first step needed for this technology and required to produce disease free plants of *L. ledebourii*. Calli characteristics and induction response vary depending on the type of explant, genotype, media components and presence of plant growth regulator in media along with other culture conditions. In agreement with our observations, MS media containing different BA concentrations in combination with various auxins (viz. 2,4-D, Picloram and NAA) was used to initiate somatic embryos in bulbous ornamental crops like *Lilium* (Bakhshaie et al. 2010a,b, Ho et al. 2006), *Narcissus* (Malik 2008, Sage et al. 2000) and *Tulipa* (Ptak and Bach 2007). A report of bulblet induction on the medium containing NAA and BA in *L. ledebourii* has been previously described (Azadi and Khosh-Khui 2007), but it did not show the regeneration of somatic embryos. In *L. longiflorum*, somatic embryogenesis from transverse thin cell layer (around 1.0 mm thickness) of pseudo-bulblets and stem sections was established on MS medium containing NAA and TDZ by Nhut et al. (2001b, 2002, 2006). All these findings clearly showed that good callus induction response is under the influence of different concentrations of plant growth regulators and explant source. Nevertheless, until now only a few reports about production of *in vitro* embryogenic callus from bulblet microscale transverse thin cell layers have been published for genus *Lilium* (Bakhshaie et al. 2010a,b).

Auxin and cytokinin are the main growth regulators in plants that regulate many aspects of plant growth and development (Kakani et al. 2009) and have been required to induce cell division and differentiation of explants during plant tissue cultures (Zeng et al. 2007). The ratio of auxin and cytokinin concentration in the culture medium is known to play a major role in the induction of shoot organogenesis or somatic embryogenesis. In general, auxins with or without low levels of cytokinins are used for induction of somatic embryogenesis, while, high levels of cytokinin alone in the culture medium induces shoot formation (Wang et al. 2008). In addition, TDZ can be substituted for auxins or the combination of auxins and cytokinins and there is evidence that it have both auxin- and cytokinin-like effects (Shen et al. 2007). In this study, it could be possible that TDZ might have fulfilled both the role of auxin and cytokinin for callus induction of *L. ledebourii*. Generally, the mechanism of action of different cytokinins possibly depend on the result of their differential uptake rate in different species, varied transportation rates to growing areas and degradation of the cytokinins through metabolic processes.

*Comparison of different culture systems*

Comparative studies between liquid, temporary immersion bioreactor and solid culture systems revealed that regeneration percentage of *L. ledebourii* through somatic embryogenesis was most efficient in solid culture. The highest percentages (94%) of embryogenic calli was observed in solid culture medium while lowest response was observed in liquid medium, compared to other culture systems (Table 1). A probable reason for this may immersions of calli into the liquid media and complete contact between the explants and the liquid medium which supplies the necessary nutrients and oxygen for growth. As previously reported the main factor, which limited the germination of somatic embryos on calluses of *L. longiflorum* is the loss of oxygen during long-term submersion in liquid medium (Nhut et al. 2006).

The results also showed that there were significant differences between the solid medium and temporary immersion system (Table 1) in which somatic embryos germinated on solid culture medium were typically of healthy appearance and formed well developed, healthy shoots and root systems as well as faster growth rates (Fig. 1C, G). Furthermore, the regeneration percentage of embryogenic calluses in the temporary immersion culture system was significantly lower than that in the solid medium (Fig. 1E), changing from 94% to 66% (Table 1). In addition, similar results were obtained in the regeneration percentage of non-embryogenic calluses in the temporary immersion bioreactor and solid culture systems (Fig. 1D, F).

It is well known that some of the benefits of temporary immersion culture systems compared to solid medium are to provide optimum growth conditions by combines the advantages of solid and liquid medium and forced ventilation through the vessel lid and, as a result, they lie in the higher multiplication rates (Escalona et al. 1999, Snyman et al. 2011). However, this could not be verified for our *L. ledebourii* *in vitro* regeneration system *via* callus development from bulblet microscale transverse thin cell layer explants cultured on solid medium supplemented with NAA and two types of cytokinins (BA and TDZ). Similar results were also reported for *Lilium* oriental hybrid 'Casablanca' by Lian et al. (2003a) who found the percent of bulblet formation was higher in solid medium than on liquid and bioreactor culture (immersion and periodic immersion in liquid media using ebb and flood). According to Teng and Ngai (1999) *Oxalis triangularis* explants of leaves, petioles and bulb scales placed in liquid-flask and bioreactor showed no response and died. Wawrosch et al. (2005) noted that the most regeneration rate without hyperhydration of the shoots was on *Charybdis* nodule explants in the semisolid basal MS medium, compared to liquid and temporary immersion culture systems. The presumed reason for faster growth in solid culture medium is thought to be due to the high rate of air exchange (Escalona et al. 1999). Therefore, under conditions of this study, it also seems that there is evidence that calluses of *L. ledebourii* were very sensitive to use for liquid culture systems and the compositions used were not suitable for desired response in liquid culture conditions (liquid and temporary immersion bioreactor). However studies by other authors suggested that such problems can be effectively controlled by the application of growth retardants (especially paclobutrazol and ancymidol) in monocots, particularly in bulbous species such as *Lilium* (Thakur et al. 2006) and *Narcissus* (Chen and Ziv 2001).

*Acclimatization*

Plantlets regenerated from somatic embryos were transferred to plastic pots containing a standard substrate mixture and maintained for 4 weeks under controlled conditions in the lab. The transplanted plantlets showed a vigorous growth and were phenotyically normal (Fig. 1H) with a survival frequency of 70% (estimated after 2 months).

**Conclusion**

In conclusion, this study shows that somatic embryogenesis and/or shoot organogenesis were simply controlled by the factors such as changes in externally added growth regulators, especially cytokinins and different culture systems. As the sole combinations of plant growth regulators in induction medium, the most suitable type of plant growth regulator was a combination of NAA with TDZ for shoot organogenesis and was a combination of NAA with BA for somatic embryogenesis in various culture systems. The use of solid culture medium is especially beneficial for micropropagation of *L. ledebourii* under the experimental conditions of this research. In future experiments the number of air exchanges, culture conditions, application of growth retardants, medium exchange frequency and type of bioreactor should be investigated in order to increase plant quality, large-scale micropropagation and lower production costs in temporary immersion systems.

**References**

Azadi P., Khosh-Khui M. (2007). Micropropagation of *Lilium ledebourii* (Baker) Boiss as affected by plant growth regulator, sucrose concentration, harvesting season and cold treatments. Electronic Journal of Biotechnology, 10: 582-591.

Bacchetta L., Remotti P.C., Bernardini C., Saccardo F. (2003). Adventitious shoot regeneration from leaf explants and stem nodes of *Lilium*. Plant Cell, Tissue and Organ Culture, 74: 37-44.

Bakhshaie M., Babalar M., Mirmasoumi M., Khalighi A. (2010a). Somatic embryogenesis and plant regeneration of *Lilium ledebourii* (Baker) Boiss., an endangered species. Plant Cell, Tissue and Organ Culture, 102: 229-235.

Bakhshaie M., Babalar M., Mirmasoumi M., Khalighi A. (2010b). Effects of light, sucrose, and cytokinins on somatic embryogenesis in *Lilium ledebourii* (Baker) Bioss. *via* transverse thin cell-layer cultures of bulblet microscales. Journal of Horticultural Science and Biotechnology, 85: 491-496.

Chen J., Ziv M. (2001). The effect of ancymidol on hyperhydricity, regeneration, starch and antioxidant enzymatic activities in liquid-cultured *Narcissus*. Plant Cell Reports, 20: 22-27.

Escalona M., Lorenzo J.C., Gonzalez B., Daquinta M., Gonzalez J.L., Desjardins Y., Borroto C.G. (1999). Pineapple (*Ananas comosus* L. Merr) micropropagation in temporary immersion systems. Plant Cell Reports, 18: 743-748.

Ghahreman A. (1997). Flora of Iran. Research Institute of Forests and Rangelands, Iran.Volume 16, No. 1944. Code 137, 001, 001.

Godo T., Kobayashi K., Tagami T., Matsui K., Kida T. (1998). In vitro propagation utilizing suspension cultures of meristematic nodular cell clumps and chromosome stability of *Lilium* × *formolongi* hort. Scientia Horticulturae, 72: 193-202.

Godo T., Matsui K., Kida T., Mii M. (1996). Effect of sugar type on the efficiency of plant regeneration from protoplasts isolated from shoot tip-derived meristematic nodular cell clumps of *Lilium × formolongi* hort. Plant Cell Reports, 15: 401-404.

Han B.H., Yae B.W., Hee J.Y., Peak K.Y. (2005). Improvement of in vitro micropropagation of *Lilium* oriental hybrid ‘Casablanca’ by the formation of shoots with abnormally swollen basal plates. Scientia Horticulturae, 103: 351-359.

Han B.H., Yu H.J., Yae B.W., Peak K.Y. (2004). In vitro micropropagation of *Lilium longiflorum* ‘Georgia’ by shoot formation as influenced by addition of liquid medium. Scientia Horticulturae, 103: 39-49.

Ho C.W., Jian W.T., Lai H.C. (2006). Plant regeneration via somatic embryogenesis from suspension cell cultures of *Lilium* × *formolongi* Hort. using a bioreactor system. In vitro Cell Development Biology – Plant, 42: 240-246.

Kakani A., Li G.S., Peng Z. (2009). Role of AUX1 in the control of organ identity during in vitro organogenesis and in mediating tissue speciWc auxin and cytokinin interaction in *Arabidopsis*. Planta, 229: 645-657.

Lian M.L., Chakrabarty D., Paek K.Y. (2003a). Bulblet formation from bulbscale segments of *Lilium* using bioreactor system. Biologia Plantarum, 46: 199-203.

Lian M.L., Chakrabarty D., Paek K.Y. (2003b). [Growth of *Lilium* Oriental Hybrid 'Casablanca' bulblet using bioreactor culture](http://apps.isiknowledge.com/full_record.do?product=WOS&search_mode=GeneralSearch&qid=7&SID=U2hfCfG@d8LkBBjINak&page=1&doc=5&cacheurlFromRightClick=no). Scientia Horticulturae, 97: 41-48.

LingFei X., FengWang M., Dong L. (2009). Plant regeneration from in vitro cultured leaves of Lanzhou lily (*Lilium davidii* var. unicolor). Scientia Horticulturae, 119: 458-461.

Malik M. (2008). Comparison of different liquid/solid culture systems in the production of somatic embryos from *Narcissus* L. ovary explants. Plant Cell, Tissue and Organ Culture, 94: 337-345.

Mii M., Yuzawa Y., Suetomi H., Motegi T., Godo T. (1994). Fertile plant regeneration from protoplasts of a seed-propagated cultivar of *Lilium × formolongi* by utilizing meristematic nodular cell clumps. Plant Science, 100: 221-226.

Murashige T., Skoog F. (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiologia Plantarum, 15: 473-497.

Nakano M., Sakakibara T., Suzuki S., Saito H. (2000). Decrease in the regeneration potential of long-term cell suspension cultures of *Lilium formosanum* Wallace and its restoration by the auxin transport inhibitor 2,3,5-triiodobenzoic acid. Plant Science, 158: 129-137.

Nhut D.T., Bui V.L., Minh N.T., Teixeira da Silva J.A., Fukai S., Tanaka M., Tran Thanh Van K. (2002). Somatic embryogenesis through pseudo-bulblet transverse thin cell layer of *Lilium longiflorum*. Plant Growth Regulation, 37: 193-198.

Nhut D.T., Bui V.L., Tanaka M., Tran Thanh Van K. (2001a). Shoot induction and plant regeneration from receptacle tissues of *Lilium longiflorum*. Scientia Horticulturae, 87: 131-138.

Nhut D.T., Bui V.L., Tran Thanh Van K. (2001b). Manipulation of the morphogenetic pathways of *Lilium longiflorum* transverse thin cell layer explants by auxin and cytokinin. In vitro Cell Development Biology – Plant, 37: 44-49.

Nhut D.T., Hanh N.T.M., Tuan P.Q., Nquyet L.T.M., Tram N.T.H., Chinh N.C., Nguyen N.H., Vinh D.N. (2006). Liquid culture as a positive condition to induce and enhance quality and quantity of somatic embryogenesis of *Lilium longiflorum*. Scientia Horticulturae, 110: 93-97.

Paek K.Y., Chakrabarty D., Hahn E.J. (2005). Application of bioreactor systems for large scale production of horticultural and medicinal plants. Plant Cell, Tissue and Organ Culture, 81 :287-300.

Paek K.Y., Hahn E.J., Son S.H. (2001). [Application of bioreactors for large-scale micropropagation systems of plants](http://apps.isiknowledge.com/full_record.do?product=UA&search_mode=GeneralSearch&qid=37&SID=V2DP5CdkPKc4ALkIFcL&page=1&doc=1&colname=WOS&cacheurlFromRightClick=no). In vitro Cell Development Biology – Plant, 37: 149-157.

Ptak A., Bach A. (2007). Somatic embryogenesis in tulip (*Tulipa* *gesneriana* L.) flower stem cultures. In vitro Cell Development Biology – Plant, 43 :35-39.

Rechinger K.H. (1989). Flora Iranica. No. 165. Liliaceae, Akademische Druck-u, Verlgsantalt, Graz, Austria, pp. 58-59.

Sage D.O., Lynn J., Hammatt N. (2000). Somatic embryogenesis in *Narcissus pseudonarcissus* cvs. Golden Harvest and St. Keverne. Plant Science, 150: 209-216.

Sankar-Thomas Y.D., Saare-Surminski K., Lieberei R. (2008). Plant regeneration via somatic embryogenesis of *Camptotheca acuminata* in temporary immersion system (TIS). Plant Cell, Tissue and Organ Culture, 95: 163-173.

Shen X.L., Chen J.J., Kane M.E. (2007). Indirect shoot organogenesis from leaves of *Dieffenbachia* cv. Camouflage. Plant Cell, Tissue and Organ Culture, 89: 83-90.

Snyman S.J., Nkwanyana P.D., Watt M.P. (2011). Alleviation of hyperhydricity of sugarcane plantlets produced in RITA® vessels and genotypic and phenotypic characterization of acclimated plants. South African Journal of Botany, 77: 685-692.

Takayama S., Akita M. (2005). Practical aspects of bioreactor application in mass propagation of plants. In: Hvoslef- Eide AK, Preil W (eds) Liquid culture systems for in vitro plant propagation, pp. 61-78. Springer, Dordrecht.

Teng W.L., Ngai Y.W. (1999). Regeneration of *Oxalis triangularis* ssp. *triangularis* from suspension cells cultured in three different systems (solid, liquid-flask and bioreactor cultures). Plant Cell Reports, 18: 701-706.

Thakur R., Sood A., Nagar P.K., Pandey S., Sobti R.C., Ahuja P.S. (2006). Regulation of growth of *Lilium* plantlets in liquid medium by application of paclobutrazol or ancymidol, for its amenability in a bioreactor system: growth parameters. Plant Cell Reports, 25: 382-391.

Tribulato A., Remotti P.C., Loffler H.J.M., Van Tuyl J.M. (1997). Somatic embryogenesis and plant regeneration in *Lilium longiflorum* Thunb. Plant Cell Reports, 17: 113-118.

Wang W.G., Zhao X.G., Zhuang G.Q., [Wang S.H](http://apps.isiknowledge.com/OneClickSearch.do?product=UA&search_mode=OneClickSearch&db_id=&SID=V2DP5CdkPKc4ALkIFcL&field=AU&value=Wang%20SH&ut=000258676000007&pos=4)., [Chen F](http://apps.isiknowledge.com/OneClickSearch.do?product=UA&search_mode=OneClickSearch&db_id=&SID=V2DP5CdkPKc4ALkIFcL&field=AU&value=Chen%20F&ut=000258676000007&pos=5). (2008). Simple hormonal regulation of somatic embryogenesis and/or shoot organogenesis in caryopsis cultures of *Pogonatherum paniceum* (Poaceae). Plant Cell, Tissue and Organ Culture, 95: 57-67.

Wawrosch C., Kongbangkerd A., Kopf A., [Kopp B](http://apps.isiknowledge.com/OneClickSearch.do?product=UA&search_mode=OneClickSearch&db_id=&SID=V2DP5CdkPKc4ALkIFcL&field=AU&value=Kopp%20B&ut=000206028000009&pos=4). (2005). Shoot regeneration from nodules of *Charybdis* sp.: a comparison of semisolid, liquid and temporary immersion culture systems. Plant Cell, Tissue and Organ Culture, 81: 319-322.

Wawrosch C., Malla P.R., Kopp B. (2001). Clonal propagation of *Lilium nepalense* D. Don, a threatened medicinal plant of Nepal. Plant Cell Reports, 20: 285-288.

Wendelbo P. (1977). Tulips and Irises of Iran and their Relatives. Botanical Institute of Iran,Tehran, Iran, 88 pp.

Zeng F.C., Zhang X.L., Jin S.X., [Cheng L](http://apps.isiknowledge.com/OneClickSearch.do?product=UA&search_mode=OneClickSearch&db_id=&SID=V2DP5CdkPKc4ALkIFcL&field=AU&value=Cheng%20L&ut=000248229200009&pos=4)., [Liang S.G](http://apps.isiknowledge.com/OneClickSearch.do?product=UA&search_mode=OneClickSearch&db_id=&SID=V2DP5CdkPKc4ALkIFcL&field=AU&value=Liang%20SG&ut=000248229200009&pos=5)., [Hu L.S](http://apps.isiknowledge.com/OneClickSearch.do?product=UA&search_mode=OneClickSearch&db_id=&SID=V2DP5CdkPKc4ALkIFcL&field=AU&value=Hu%20LS&ut=000248229200009&pos=6)., [Guo X.P](http://apps.isiknowledge.com/OneClickSearch.do?product=UA&search_mode=OneClickSearch&db_id=&SID=V2DP5CdkPKc4ALkIFcL&field=AU&value=Guo%20XP&ut=000248229200009&pos=7)., [Nie Y.C](http://apps.isiknowledge.com/OneClickSearch.do?product=UA&search_mode=OneClickSearch&db_id=&SID=V2DP5CdkPKc4ALkIFcL&field=AU&value=Nie%20YC&ut=000248229200009&pos=8)., [Cao J.L](http://apps.isiknowledge.com/OneClickSearch.do?product=UA&search_mode=OneClickSearch&db_id=&SID=V2DP5CdkPKc4ALkIFcL&field=AU&value=Cao%20JL&ut=000248229200009&pos=9). (2007). Chromatin reorganization and endogenous auxin/cytokinin dynamic activity during somatic embryogenesis of cultured cotton cell. Plant Cell, Tissue and Organ Culture, 90: 63-70.