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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 [6-Benzyladenine (BA) and Thidiazuron (TDZ)] were studied on the *in vitro* plant regeneration of *L. ledebourii*. 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 percentage (94%) of embryogenic callus was 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 rather than solid culture systems. Seventy percent of the plantlets were successfully acclimatized to *exvitro* conditions and were phenotypically similar to the mother plants.

Keywords: bioreactor, embryogenic callus, *Lilium ledebourii*, liquid culture, organogenesis.

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Abbreviations

BA 6-Benzyladenine

MS Murashige and Skoog (1962)

NAA α-Naphthaleneacetic Acid

TDZ Thidiazuron

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 (7, 30, 42). The plant whole height is 50–150 cm that begins to blossom around the middle of July in the seventh year of its growth period. Leaves are erect, narrow, alternate, lanceolate, 10–14×1–2cm, sessile and 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 (3, 4, 7).

The naturally low rate propagation by seed and rapid eradication of *L. ledebourii* (1, 3, 4, 20) 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 (27, 28, 35); 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 (26). More efficient procedures for plant regeneration from long-term maintenance of cell suspension cultures of *L. formosanum* (22), cell suspension culture of *L. longiflorum* (38) and suspension culture-derived protoplasts of *L. formolongi* (9, 19) 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 (8, 12, 15, 16, 27, 28, 35, 37).

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*, *L. nepalense* and *L. pumilum* (2, 4, 10, 11, 13, 17, 24, 25, 41) while in other studies on *L. ledebourii*, *Lilium* × *formolongi* and *L. longiflorum* (3, 4, 12, 23, 25, 26), cytokinins were shown to stimulate somatic embryogenesis and plant regeneration.

Up until now no protocols of *L. ledebourii* reproduction in liquid and temporary

immersion bioreactor systems have been elaborated (3, 4, 20). In the presented research work, for the first time induction of calli in *L. ledebourii* on the basal Murashige and Skoog (21) (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 induction, reported by Bakhshaie et al. (3).

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 16h 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. (32). The immersion frequency in the temporary immersion system was 5 min every 6 h.

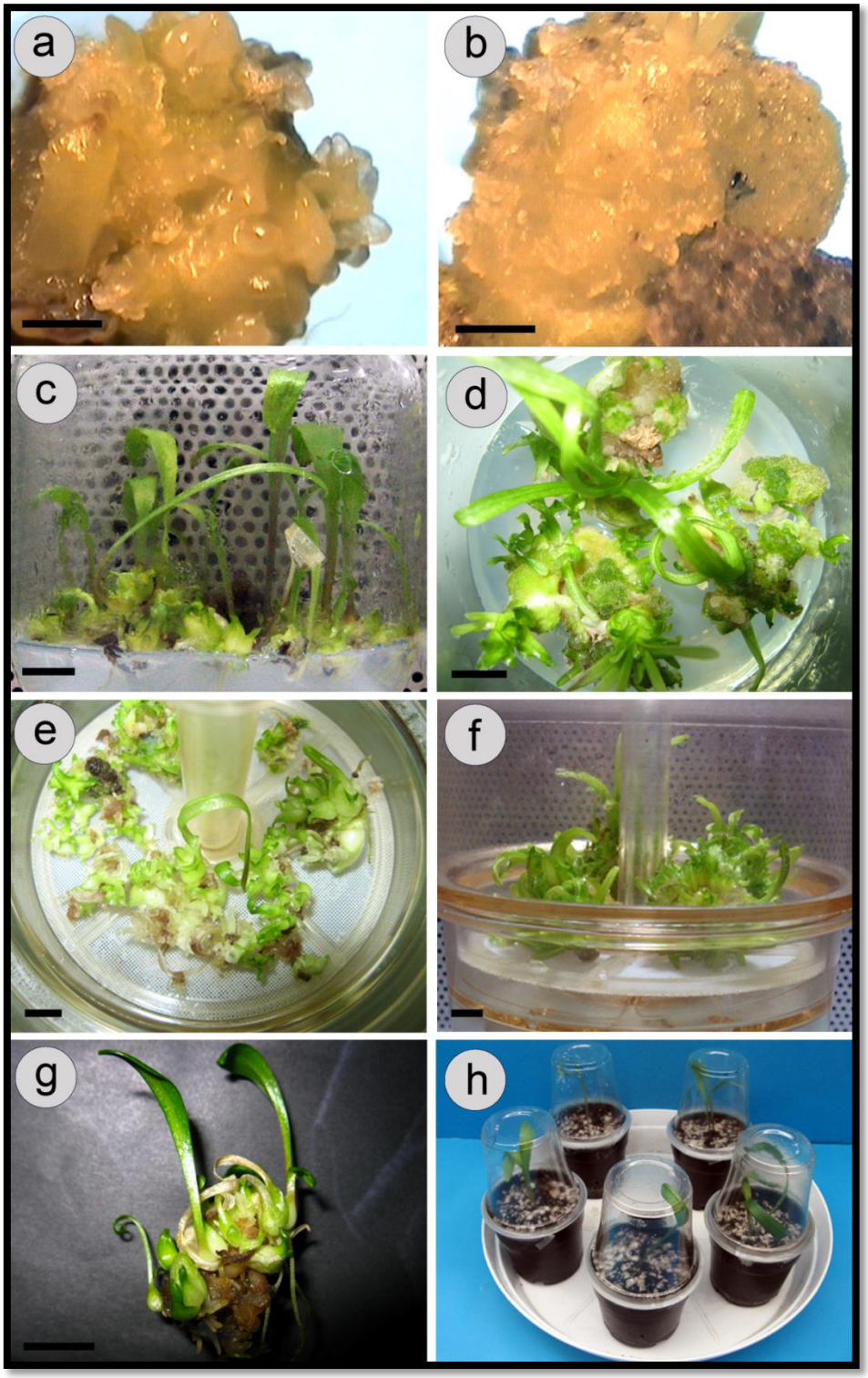

Figure 1. Micropropagation of *Lilium ledebourii*. a Induction of type I calli on solid MS medium containing 0.54 µM NAA and 0.44 µM BA in the darkness, b Induction of type II calli on solid MS medium supplemented with 0.54 µM NAA and 0.45 µM TDZ in the darkness, c Regenerated plantlets from type I calli on a solid hormone-free MS medium, d Shoot organogenesis from type II calli on a solid MS medium free of hormones, e Plantlets with shoots and roots from type I calli produced in a RITA® bioreactor, f Shoot organogenesis from type II calli formed in a RITA® vessels, g Plantlets were regenerated from type I calli with roots on the base of shoots, h Plants in pots after 2 weeks of growth in a controlled environment. The *Bars* in a and b are represent 1 mm, whereas those in c, d, e, f and g are scaled in 1 cm.

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 $P \leq 0.05$.

Results

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).

Table 1. Effects of culture systems and different cytokinins tested on survival percentage of calli and *in vitro* morphogenesis in *Lilium ledebourii*

Culture system	Growth regulators (μM)	Survival percentage of calli [a]	Morphogenesis
Liquid medium	NAA 0.54 + BA 0.44	0 e	– [b]
	NAA 0.54 + TDZ 0.45	0 e	–
Bioreactor	NAA 0.54 + BA 0.44	66 c	Somatic embryogenesis
	NAA 0.54 + TDZ 0.45	33 d	Shoot organogenesis
Solid medium	NAA 0.54 + BA 0.44	94 a	Somatic embryogenesis
	NAA 0.54 + TDZ 0.45	72 b	Shoot organogenesis

Means in the column followed by different letters are different according to Duncan's multiple range test at $P \leq 0.05$.

a The survival percentage of calli was determined by dividing the number of surviving explants by the total number of cultured explants and multiplying by 100.

b Data not available due to the failure of regeneration from calluses cultured in the liquid medium.

Abbreviations: BA - 6-benzyladenine; NAA - a-Naphthaleneacetic acid; TDZ – thidiazuron.

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 percentage (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 (26).

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).

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 laboratory. The transplanted plantlets showed a vigorous growth and were phenotyically normal (Fig. 1h) with a survival frequency of 70% (estimated after 2 months).

Discussion

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* (3, 4, 12), *Narcissus* (18, 31), and *Tulipa* (29). A report of bulblet induction on the medium containing NAA and BA in *L. ledebourii* has been previously described (1), 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 according to Nhut et al. (23, 25, 26). 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* (3, 4).

Auxin and cytokinin are the main growth regulators in plants that regulate many

aspects of plant growth and development (14), and have been required to induce cell division and differentiation of explants during plant tissue cultures (43). 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 (39). In addition, TDZ can be substituted for auxins or the combination of auxins and cytokinins. There is also evidence that it has both auxin- and cytokinin-like effects (33). 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.

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 combining 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 (6, 34). 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. (15) who found that 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 (36) *Oxalis triangularis* explants of leaves, petioles and bulb scales placed in liquid-flask and bioreactor showed no response and died. Wawrosch et al. (40) 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 considered due to the high rate of air exchange (6). 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* (37) and *Narcissus* (5).

Conclusion

Somatic embryogenesis and/or shoot organogenesis were/was 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 to lower production costs in temporary immersion systems.

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