RESEARCH ARTICLE

Eurasian Journal of Medical and Biological Sciences
AN INTERNATIONAL JOURNAL
Eurasian J Med Biol Sci **2(1): 23-30**

A Successful Encapsulation Technique for the Production of Synthetic Seeds for *Lilium candidum* L. Grown Naturally in Turkey

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Received : 02/03/2021 Revised : 13/04/2022 Accepted : 15/04/2022

ABSTRACT: *Lilium candidum* L. (white lily) is in danger of extinction due to the destruction of its natural habitat in Turkey and the removal of its tubers from nature for export. For this reason, it is very important to carry out biotechnological studies with *L. candidum*, which is an important medicinal and ornamental plant. *In vitro* techniques can provide the opportunity to preserve plant genetic resources in different forms and different genetic features (synthetic seed). With these techniques, it is possible to reproduce plant species rapidly in danger of extinction and transfer them to their natural environments with *in vitro* and *ex situ* conservation studies. In this study, we aimed to establish a suitable protocol for the production of synthetic seeds in *L. candidum.* Accordingly, regeneration was achieved by using the stem bulbils of the plant. An effective encapsulation technique has been created for the production of synthetic seeds in *L. candidum*, obtained from its calli by regeneration. The calculated callus development and embryogenesis rates of encapsulated calli were higher than single ones, 92.5%-88.6%, and 90.4%-77.1% respectively. Our protocol turned out to be successful for synthetic seed production as well as large-scale *in vitro* clonal propagation of *L. candidum.*

Keywords: Ca-alginate, commercial production, white lily, artificial seed

INTRODUCTION

Biodiversity refers to the richness of genes, species and ecosystems in a region. Plant diversity forms the basis of natural resources, which have an indispensable place in meeting basic needs, primarily food. Wild species also make an important contribution to the field of medicine. Wild plants originate half of the drugs used in medicine and approximately 80% of the world's population uses plants as the primary source of drugs (Giam et al., 2010; Reed et al., 2011). *Lilium candidum* L. an ornamental plant, is one of them. *L. candidum* is an Eastern Mediterranean herb distributed in Turkey, Lebanon, Syria, Palestine, the Greek Islands, and the Balkans (Davis, 1984; Altan et al., 2010). The natural growing places of *L. candidum* in Turkey are Aydın, Muğla, Antalya, İzmir, Manisa, Balıkesir and Denizli, as we observed in line with our project (Altan et al., 2021). Although *L. candidum* has been used for various purposes since the early ages, especially in the middle ages, it took its place in the churches as a symbol of cleaning and purity due to its scent and white flowers. This plant, which is of great medical importance is also in great demand in the treatment of boils, skin spots, and burns as a diuretic and expectorant. In addition, it is stated that extracts obtained from the roots of this plant provide benefits in removing edema (Bown, 1995). Due to its fragrance, this plant is used in perfume production in the cosmetic industry (Baytop, 1984; Chopra et al., 1986).

As a result of cultural and sociological changes, cities around the world are spreading to large areas. Therefore, the importance of maintaining green areas is increasing in urban areas. The demand for ornamental plants has increased worldwide in this direction. Plants that have low economic value compared to other species but can be used in cosmetics, pharmaceuticals, food, and chemical industries also have wide usage potential in many fields including landscape architecture. While some of these species can be easily produced, some cannot be, due to some problems in their production. Due to these difficulties in their production, it is known that many valuable ornamental plants have come to extinction. *L. candidum* is a perennial herbaceous plant used in the pharmaceutical perfumery industry and widely grown as an ornamental plant (Özen et al., 2016). The species of this plant is endangered by overgrowing and the destruction of its habitat from various conditions such as overgrazing, irregular harvesting, global warming and pollution, as well as various natural phenomena such as less seed formation, dormancy, poor germination rate (Özen et al., 2016; Gantait et al., 2017). Depending on the species, production materials such as seeds, plant cutting, tubers and bulbs are used in ornamental plant breeding. World markets and the pharmaceutical industry demand "standard" products with high active ingredient quantity and quality. Today, it is not possible to obtain sufficient norm and quality products with the collection of natural plants, and it is necessary to ensure the production of these plants with rapid reproduction techniques (Bayram et al., 2010). One of the alternative application methods accepted in recent years is to use *in vitro* techniques for propagating ornamental plants. *In vitro* techniques also offer great advantages for healthy and fast production. These include the rapid production of typical genotypes, early selection *in vitro* for durability, the production of haploid plants, obtaining disease-free plants, the production and selection of mutants, rejuvenation for classical vegetative propagation,

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preservation of genetic diversity, gene transfer by DNA technology, protoplast culture and *in vitro*, somatic hybridization, somatic embryo formation and synthetic seed production (Bhojwani and Razdan 1983; Vidalie, 1986). Synthetic seed production supports the large scale production of plant material that can be employed in different laboratory conditions in *in vitro* culture systems. The synthetic seeds offer a lot of benefits such as micropropagation and conservation of gene resources, transportability, storability, reduced size of propagules (Gantait et al., 2012). In this method, growth regulators are added to the nutrient medium to induce embryos in calluses. In many plants, somatic embryogenesis method (Figure 1) is used in making synthetic seeds (Özcan et al., 2001) The main purpose of encapsulation technology is to protect, store and transport somatic embryos. This technology facilitates the transfer of plants produced *in vitro* to the land or greenhouse (Soneji et al., 2002). Biotechnological studies have increased on *Lilium* spp. in recent years (Bakhshaie et al., 2016). Micropropagation and *in vitro* regeneration have been reported in *L. candidum*, but synthetic seed production has never been reported. In the current study, the effective synthetic seed protocol were optimized for *L. candidum*, and the synthetic seeds were also obtainedfrom calli derived from *L. candidum* leaf explants. It is generally accepted that, a whole *in vitro* regeneration and/or germination procedures require to be set up for each explant type of each plant species, and for each *in vitro* propagation step, in terms of culture conditions such as regeneration and/or germination medium, light intensity, temperature, explant proposal, concentration and combination of plant growth regulators. It is very important to develop an effective vegetative production method for classical ornamental plants, which have difficulties in production and which are of medical importance, apart from classical production methods. In this way, both the increasing needs can be met and the extinction of the generations of these species can be prevented. In this study, rapid and effective production of *L. candidum* with synthetic seed is aimed rather than classical methods.

MATERIALS AND METHOD

Plant material

The natural populations of *L. candidum* were collected from Izmir (Nif Mountain). The legal authorization letter for sample collection was obtained from Republic of Turkey Ministry of Agriculture and Forestry and all collected samples were taxonomically identified.

Surface Sterilization and In Vitro Culture Establishment

The *L. candidum* bulbils (Figure 2a) were treated with 70% ethanol for 5 min, respectively 20% and 10% commercial bleach (Domestos®) for 10 min, then they were rinsed in distilled water at least three times (Özüdoğru et al., 2011). After surface sterilization, the bulbils were transferred to semi-solid MS (Murashige and Skoog 1962) medium supplemented with 4.44 µM 6-Benzylaminopurine, 20 g L^{-1} sucrose and 7 g L^{-1} agar (pH 5.8) (Tokgöz and Altan, 2020).

Callus Induction Conditions

For callus induction, ~ 0.75×0.5 cm leaf fragments derived from *in vitro* regenerated bulbils (Figure 2b) were transferred to MS medium supplemented with 10.7 µM 1-Naphthaleneacetic acid (NAA), 20 g L^{-1} sucrose and 7 g L^{-1} agar (pH 5.8) (Tokgöz and Altan, 2020). The calli obtained were used for control purposes in the study.

Synthetic seed production

The nearly 150 mg of separated callus cells were added to 10 mL of a 3% sodium alginate solution (low viscosity) in Ca^{++} free MS liquid medium (Murashige and Skoog, 1962). To obtain Ca-alginate beads, the alginate solution including callus cells were dropped to MS medium containing 100 mM $CaCl_2$ using a sterile pipette for polimerization (Souza et al., 2017). The Ca-alginate beads including the callus cells were washed with sterile dH_2O to prevent them from sticking each other (Figure 3a).

Calli Multiplication and Somatic Embryo Induction

For *in vitro* multiplication of *L. candidum* callus cells, the beads were transferred to OM (Olive Medium) (Rugini, 1984) supplemented with 4.44 µM 6-benzylaminopurine (BAP), 20 g L^{-1} sucrose, 3.5 g L^{-1} agar and 1.5 g L^{-1} phytagel (pH 5.8). After four weeks incubation, the synthetic seeds were transferred to OM medium supplemented with 21.4 µM NAA, 20 g L^{-1} sucrose and 7 g L^{-1} agar (pH 5.8). After conversion (Figure 3b), the somatic embryos (Figure 3c, 3d) were obtained from transferring OM supplemented with 10.7 µM NAA, 20 g L^{-1} sucrose and 7 g L^{-1} agar (pH 5.8).

In Vitro Culture Conditions

The *in vitro* culture conditions were adjusted to 25±2°C, under a 8 hours dark / 16 hours light photoperiod, with light provided by cool daylight fluorescent lamps (50 $\mu mol^{-1}m^{-2}s^{-1}$).

Data Analyses

The calli multiplication of *L. candidum* synthetic seeds and the somatic embryogenesis data were calculated as percentages values. All data were collected after four weeks incubation at standard culture conditions described above. The statistical analysis of the non-parametric data was performed by means of the test for homogeneity rates, and the differences obtained in treatments were chosen using non-parametric statistical test (Marascuilo and Mcsweeney, 1977). Separate data were exposed to ANOVA, monitored by the least significant difference test at $P \leq 0.05$ to compare means.

RESULTS and DISCUSSION

In this study, *in vitro* callus culture initiation was achieved by using leaves derived from *L. candidum* bulbils (Figure 2a) and the calculated regeneration percentage was approximately 89.5% (Tokgöz and Altan, 2020) on regeneration medium described above after four weeks incubation (Figure 2b). The ~ 0.75×0.5 mm leaves fragments derived from *in vitro* grown healthy *L. candidum* bulbils were used for callus induction. The callus formation frequency was 100% of all explants, depending on 10.7 µM 1-NAA as auxin sources and the smooth whitish-yellow colored calli were obtained from widening and hardening leaf fragments. Cell proliferation causing these great callus formation rates, which induced from injured fragments of leaves may have been resulted from accumulation of NAA at injured side of leaf segments. These soft smooth whitish-yellow colored calli were used as explant source for synthetic seeds production. The maximum cell multiplication rates of calcium alginate beads containing *L. candidum* calli were obtained from OM proliferation medium described above and calculated germination percentage was only 10% (Figure 4a) after 4 weeks incubation at standart culture conditions described before, however, after trasferring to OM medium medium supplemented with 21.4 µM NAA, the germination percentages of calcium alginate beads was calculated as 100% (Figure 4b) after an additional four weeks of incubation. The callus development and embryogenesis rates of encapsulated calli were statistically significantly different from single callus cells. When the calculated callus development and embryogenesis rates of encapsulated calli were 92.5% and 90.4%, these rates for single calli were 88.6% and 77.1% respectively (Table 1). The gelling material has heterogeneous polymers having functional properties and their successes as usage of synthetic seeds depend on plant material and methodology optimization for each treatment (Thu et al., 1996). Synthetic seed production by using callus tissues in a suitable gelling material such as calcium alginate can be a useful tool for large-scale multiplication of plant material (Kumar and Loh, 2012). The mineral nutrients presence promoted the callus development and embryogenesis of synthetic seeds in *L. candidum.* This result provided a critical point for mineral nutrient significance on the viability, development and embryogenesis of encapsulated calli. That result were confirmed by mineral nutrients addition to the capsule and similar results have also been observed in related studies (Cangahuala-Inocente et al., 2007; Majd et al., 2010; Souza et al., 2017). All proliferated calli from conversion of calcium alginate beads produced through indirect embryogenesis and the healthy emblings were obtained from these embryos (Figure 4c, d). At first, induced calli produced pale yellowish to light green globular to fusiform pro-embryoids as well as prototype of plantlets. Globular and/or fusiform embryoids developed from soft smooth whitish-yellow colored calli on OM medium supplemented with 10.7 µM NAA. These embryoids were subcultured to obtain further growth on same medium.

To obtain synthetic seeds use of nodal regions, bulbs, and in vitro propagated shoots are made. Synthetic seed formation process proceeds as callus, pre-embryo formation, somatic embryo formation, embryogenesis and plant regeneration (Arnold and Eriksson, 1981; Reddy et al., 2012). In the literature, the report for synthetic seed production in *Lilium* spp is very limited (Kaviani, 2010), as in other bulbous plants. However, in our study, a very successful protocol was provided. In this protocol, synthetic seed production was ensured in calli developed from the shoots obtained from stem bulbs. Somatic propaguller (embryo or buds) used in synthetic seed production are covered with hydrogel. For this purpose, the most commonly used viscose, low toxic and fast gelling agent, alginate is preferred. Although there are many gel agents such as potassium alginate, agar, gelrit and sodium pectate, but calcium alginate has been suggested to be more suitable than other gel agents (Redenbaugh et al., 1987; Nongdam, 2016). By controlling the chemicals in the culture medium, a single plant can be obtained from propaguli (Reddy et al., 2012). In *Manihot esculenta* Crantz, pieces taken from nodal areas and shoot ends were covered with 3% sodium alginate and synthetic seeds were prepared (Danso and Ford-Lloyd, 2003). In the study with *Malus pumila* Mill (M26), the first five leaves were used for regeneration of shoots and the shoot ends obtained from them were encapsulated using 2% sodium alginate and propogated into synthetic seeds (Sicurani et al., 2001). Somatic embryos are the most commonly used products in synthetic seed technology (Özcan et al., 2001), but only a few studies on the production of synthetic seeds with somatic embryos in flower bulbs have been reported. Winkelmann et al., (2004) created somatic embryos using liquid culture method in *Cyclamen persicum.* The researchers also performed synthetic seed formation by covering somatic embryos with alginate capsules. Pradhan et al., (2016) observed synthetic seeds in *Cymbidium aloifolium* by covering protocorms with calcium alginate. Calcium alginate was also used in our study. In addition, as in our study, researchers used BAP and NAA as a growth regulator. Although there are studies indicating that storage at 4 °C is the most suitable for synthetic seed preservation, there are studies reporting that storage at higher temperatures is better (Gantait et al., 2012; Haque and Ghosh, 2014; Haque and Ghosh, 2016; Bhattacharyya et al., 2018). In our study, successful shoot formation in synthetic seeds at room temperature (25±2°C) was observed after four weeks. Yücesan et al., (2014) used bulbils as propagules to produce synthetic seeds in *Muscari armeniacum*. The researchers stated that the germination rate of synthetic seeds was 95%. In current study, the germination percentages of calcium alginate beads was calculated as 100% (Figure 4b) after an additional four weeks of incubation.

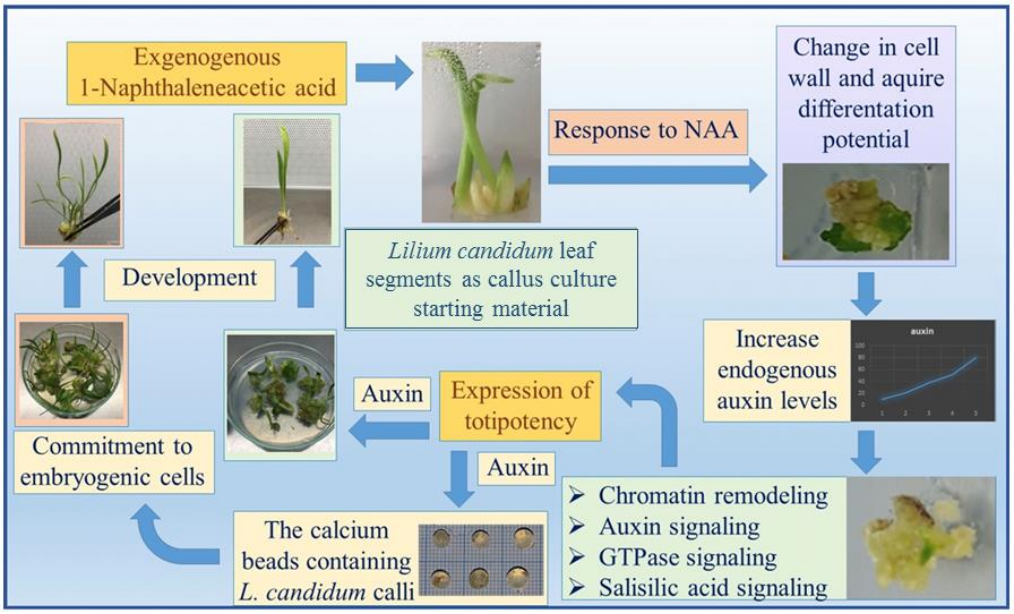

Figure 1. *The possible molecular regulation mechanism for somatic embryogenesis of L. candidum calli* (The possible functions of plant growth regulators expressed in the present figure is taken from Elhiti et al., 2013).

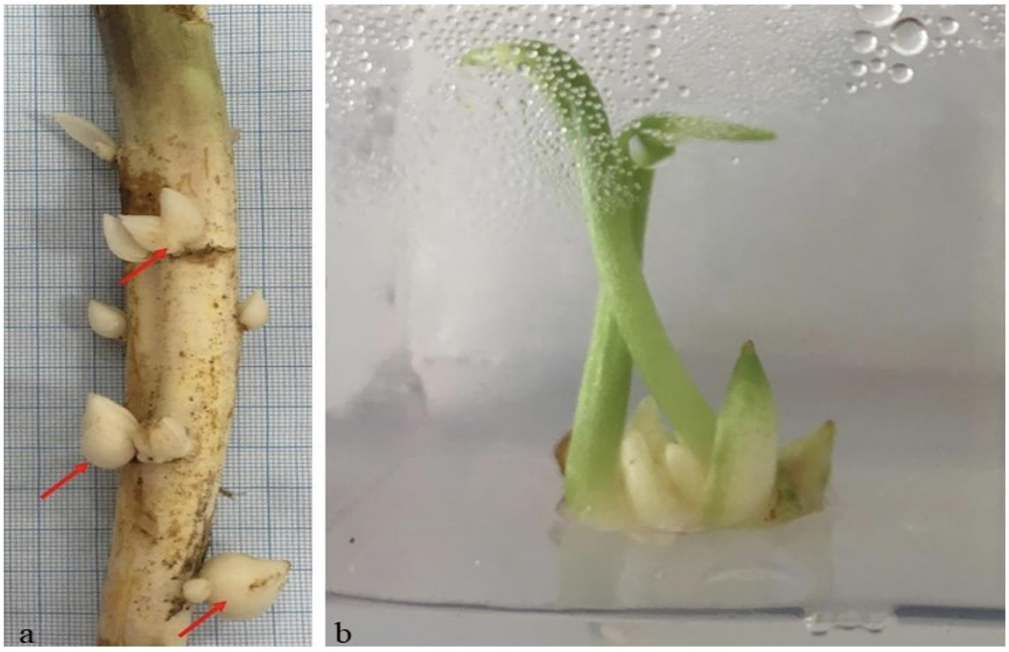

Figure 2. (a) *Bulbils used for in vitro culture initiation (the arrows indicated bulbils which were used for culture establishment)* ***(b)*** *In vitro regenerated bulbils.*

Table 1. *Comparison of single and encapsulated calli of L. candidum for callus development index, regeneration and embryogenesis rates.*

	Development Rate* **(% ± SE***)**	**Embryogenesis Rate**** **(% ± SE)**
Single Calli (as control)	$88.6 \pm 2.34^{B****}$	77.1 ± 2.17^{b}
Encapsulated Calli	92.5 ± 1.71^{A}	90.4 ± 1.50^{a}

*Production of healthy, smooth whitish-yellow colored calli from starting calli on OM medium supplemented with 21.4 µM NAA

** Production of pale yellowish to light green globular to fusiform pro-embryoids as well as prototype of plantlets OM medium supplemented with 10.7 µM NAA

***Standart error

**** SPSS statistical analyzes were performed with X^2 test and ANOVA (LSD test, P≤0.05)

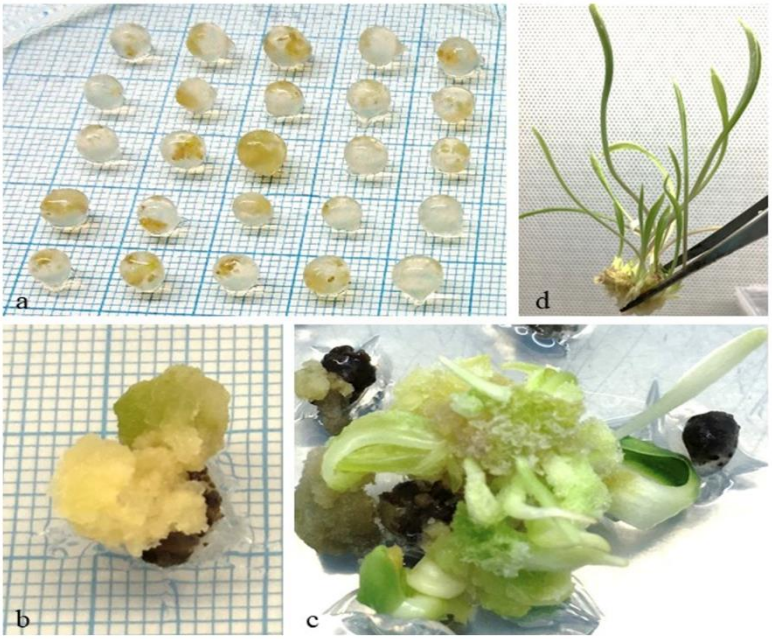

Figure 3. ***(a)*** *Ca-alginate beads containing L. candidum callus cells, cell multiplication* ***(b)*** *and somatic embryogenesis* ***(c)*** *of L. candidum synthetic seeds, in vitro grown embling* ***(d)*** *derived from L. candidum synthetic seeds.*

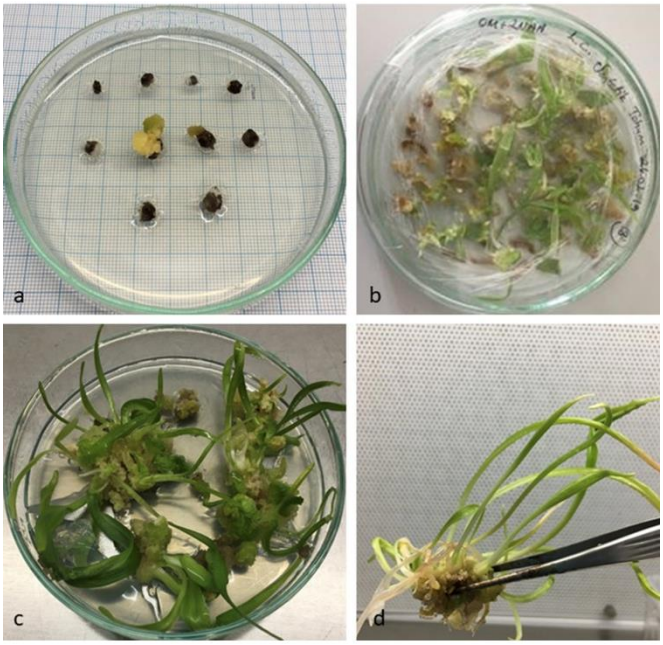

Figure 4. *Conversion of calcium beads containing L. candidum calli after 4 weeks incubation* ***(a)*** *on OM proliferation medium after an additional 4 weeks of incubation* ***(b)*** *somatic embryogenesis* ***(c)*** *of L. candidum synthetic seeds, in vitro grown embling,* ***(d)*** *derived from L. candidum synthetic seeds.*

CONCLUSION

Today, it is possible to produce many plants quickly in a laboratory environment with tissue culture methods. At the same time, the results obtained from tissue culture studies are used in synthetic seed production, somatic hybridization and conservation of gene resources. It is important to emphasize that the encapsulation technology is one of the complementary alternatives for the protection of ornamental plants. In this study, an effective synthetic seed production was provided in *L. candidium.*

ACKNOWLEDGEMENTS

This study was supported by the Scientific and Technological Resarch Council of Turkey (TUBİTAK) under Grant No: TOVAG-217O009. Authors thank to Assoc. Prof. Dr. Hasan Yıldırım and Assist. Prof. Dr. Ademi Fahri Pirhan for providing plant material.

CONFLICT OF INTEREST

No conflict of interest was declared by the authors.

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