

Efficient Approaches to *in vitro* Multiplication of *Lilium candidum* L. with Consistent and Safe Access throughout Year and Acclimatization of Plant under Hot-Summer Mediterranean (Csa Type) Climate

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Abstract

Lilium candidum L. is one of the most fragrant plant species among Liliaceae. The indigenous and natural populations of *L. candidum* are significantly affected by fast increasing anthropological population pressure and increased carbon fuel pollution. There is a necessity for development of a micropropagation protocol to serve in effective manner for both commercial multiplication and protection of the plant. The study targeted to develop a strategy for effective *in vitro* plant propagation system using static liquid culture, seismomorphogenic treatments with mixed liquid culture and semi solid culture medium using single scale explants. The former two techniques failed to regenerate new bulblets effectively whereby the later technique induced 100% regeneration on all treatments. Maximum number of bulblets regenerated on 0.27 μ M Thidiazuron + 1.08 μ M Naphthalene acetic acid. The scale explant could be recultured and regenerated on the mother culture medium for six cycles. The daughter bulblets regenerated on Murashige and Skoog medium containing both 0.18 or 0.27 μ M TDZ + 1.08 μ M NAA were successfully rooted using 1/2 \times MS medium containing 2.45 μ M indole butyric acid. Regardless of rooting behavior 100%, acclimatization was noted on all bulblets in the greenhouse. These plants are showing continuous flowering since last four years under hot-summer Mediterranean (CSa type) climate field conditions of Tekirdag province of Thrace region in Turkey.

Keywords: *in vitro*; *Lilium candidum*; rooting; single scale bulb

Introduction

Liliaceae family incorporates around 250 genera and about 3000 species widespread all over the world. Genus *Lilium* L. contains about a hundred species distributed in between 10-60 latitude in the northern hemisphere (Dostal, 1989). There are nine species of *Lilium* that are spread throughout Turkey of which three are endemic (Tubives, 2017). These perennial bulbous plants have top-notch magnificence and high mercantile commercial importance (Comber, 1949; Davis, 1985; Mabberley, 1990; Sharma *et al.*, 1996; Siljak-Yakovlev *et al.*, 2003).

Lilium candidum L. (Liliaceae family) is a bulbous geophyte with 90-150 cm tall erect green plants. It bears 3-20 large lanceolate leaves and trump shaped pure white very fragrant attractive flowers (Mouterde, 1966; Feinbrun Dothan and Danin, 1991; Blamey and Grey-Wilson, 1993) and has large commercial and mercantile value. Flowering occurs in May (Tubives, 2017) in Turkey, where it has a largely dispersed and small number of acidic soil habitats distributed on hot, dry steep limestone rocky mountainous

slopes and ledges in Antalya, Aydın, Balıkesir and Muğla provinces of Mediterranean low evergreen tree and shrub habitats (phrygana and maquis) at 100-1,200 m asl, and flowers in May and early June (Temeltaş, 1999; Ozen *et al.*, 2012; Tubives, 2017). The plant is considered to have originated in an area extending from Turkey, Balkan countries, Lebanon, Syria, Palestine including some parts of Israel and Persia (Mouterde, 1966; Polunin, 1987; Feinbrun-Dothan and Danin, 1991; Blamey Grey-Wilson, 1993; Khan *et al.*, 2010; Tubives, 2017).

The plant was abundantly gathered from the wild and propagated and planted in gardens close to houses for fragrance. This helped the spreading of the plant in the Mediterranean region (Mouterde, 1966; Blamey and Grey-Wilson, 1993) abroad. The conservation of wild species are particularly important (Barazani *et al.*, 2008) and the fact that it holds a rare potential as a new ornamental crop, *L. candidum* is considered an endangered, with a high priority for conservation. *Lilium* species could be propagated through sexual or asexual means; however, the plants have limited seed production in the wild (Temeltaş, 1999; Uysal

and Kaya, 2013; Ocak *et al.*, 2014) Moreover, the propagation by seeds is very uncertain, as the seeds have high dormancy and it may take 4-5 years to develop bulbs of desirable size to induce flowers from them (Khawar *et al.*, 2005; Sevimay *et al.*, 2005). Over and above, use of seeds is only desirable when there is need to maintain diversity for breeding purpose. However, they are not desirable for commercial production of ornamental plants (Bakhshaie *et al.*, 2016). Furthermore, the developing bulbs may also suffer from uncertain biotic and abiotic stresses during early stages of growth.

No proper field propagation system is available. There are few studies pertaining to *in vitro* (Khawar *et al.*, 2005; Sevimay *et al.*, 2005) and *ex vitro* multiplication of the plant in Turkey (Temeltaş 1999; Özen *et al.*, 2012; Uysal and Kaya, 2013; Ocak *et al.*, 2014).

High urbanization, increased industrial activities, increased fossil fuel consumption related CO₂ pollution, has created an extra constraint and pressure on natural resources (IPCC, 2007; Stocker *et al.*, 2010) that has resulted in creation of pressure and threat to natural flora at their habitats. Establishment of micropropagation protocols and establishment of *L. candidum* have great importance. Tissue culture methods could help in propagation of plants on commercial scale, breed new cultivars with introduction of sought after desired characteristics like resistance to abiotic or biotic stresses desired flowering time, improved fragrances and similar other flower-related characteristics (Granados-Sánchez and Castañeda-Pérez, 1998; Gutterman and Chauser-Volfson, 2007).

A possible alternative to accelerate propagation could be *in vitro* micropropagation, singly or by integrating it with traditional propagation techniques. *In vitro* culture methods provide an alternative way for rapid and continuous supply of homogenous plant material to field cultivation and may serve to shorten the period between juvenile and physiologic maturity to induce flowering (Cristiano *et al.*, 2016).

Thrace is a tourist region in Turkey, but there is no report of growing of this plant in this region under wild or domesticated conditions on slight saline soils in spite of the high economic returns. The aim of this study was to establish a feasible protocol for rapid *in vitro* multiplication ensued by introduction of this plant under field conditions for greater economic gains.

Materials and Methods

Bulbs of *L. candidum* were obtained from natural habitat of Dalyan, Muğla province in the Mediterranean region of Turkey. Soon after collection, they were washed in running tap water to remove mud and dirt if any followed by their drying and wrapping in paper bags. Subsequently, they were brought to the laboratory and incubated at 8 °C for 6 weeks. The sterilization of bulbs started by treating the bulbs with a dip in 96% ethanol for 15-30 seconds. This was followed by sterilization in 50% commercial bleach (2.5% NaClO) for 10 min and then rinsed 3 × 5 min in sterilized distilled water. Thereafter, intact single scales (isolated from the bulbs) were cultured on stationary liquid culture, given seismomorphogenic treatments with mixed liquid culture

or semi solid [gelled with 7 g/l agar (Duchefa)] MS medium (Murashige and Skoog, 1962) - used as control or MS medium containing 5 combinations each of 0.18 or 0.27 μM thidiazuron (TDZ) + 0.0, 0.54, 1.08, 2.16 and 3.24 μM Naphthaleneacetic acid (NAA) in sterilized baby food culture jars (Product Code V8630 - Magenta B-cap) with 3 g/l activated charcoal and 30 g/l sucrose (Fig 1a). All cultures were incubated at ± 4 °C for 9 days initially. Thereafter, these cultures were treated at 24±1°C less than 16 h light photoperiod (DAIHAN Growth/Environmental Chamber/ South Korea).

Rooting

The 100 randomly selected healthy and well developed bulblets regenerated in two experiments on MS medium containing 0.18 μM TDZ + 1.08 μM NAA and 0.27 μM TDZ + 1.08 μM NAA were induced rooting by transferring them to ½ × MS medium containing 2.45 μM IBA for 21 d with 6 replications. Every treatment contained 100 *in vitro* regenerated plants (four plants per pot). A comparison between the rooting behaviors of the two differently regenerated bulblets was also performed. The bulblets were cultured on MS medium containing 30 g/l sucrose for 28 days followed by their vernalization on MS medium containing 60 g/l sucrose with 30 days incubation at 5±1 °C in dark in agreement with (Waters and Wilkins, 1967). The plants in all pots were uprooted carefully without damaging the root apparatus to note morphologic changes on bulb size and roots. These were washed tenderly in tap water at room temperature to remove any agar gel adhering to the roots. Thereafter, all bulblets were transferred to 1 liter pots containing 0.850±20 ml peat and placed in growth chamber with 80% humidity and 12 h light (35 μmol photons m⁻² s⁻¹) photoperiod for ~ 4 weeks to acclimatize them to the external environment. Subsequently, the acclimatized plants were moved to the greenhouse. Peat moss held 10 times moisture of its dry weight in water with pH of 6.15 and electrical conductivity (EC) of 0.13 dS m⁻¹, with 69% (v/w) porosity, and allowed high water absorption with low bulk density of 0.15 mg m⁻³. After observance of growth signs on the plants, the humidity was gradually reduced to 40% in 15 days time. Subsequently, the pots were transferred to a shady place and then to open fields; where they were watered (100 ml/plant) for first four weeks every day. Thereafter, the watering was done, subject to the plant needs and meteorological factors affecting plant growth.

Meteorological observations

Average, maximum and minimum temperature remained 8.0-25.3 °C respectively (Table 1). Minimum high temperature was noted during January and the maximum temperature was noted during August 2012. Average, maximum and minimum high temperature remained 17.5-34.6 °C respectively. Minimum high temperature was noted during December and the maximum high temperature was noted during July 2014. Average, maximum and minimum low temperature remained -0.8 - 17.1 °C respectively. Minimum temperature was noted during December and the maximum temperature was noted during July. Average, maximum and minimum precipitation remained 6 mm - 107.7 mm respectively.

Minimum precipitation was noted during January and the maximum precipitation was noted during July 2014. Average, maximum and minimum relative humidity remained 73.0-89.1% respectively. Minimum relative humidity was noted during July and the maximum relative humidity was noted during December 2014.

Soil characteristics

The 15 random soil samples were collected from 0 - 20 cm depth as described by (Jackson 1965). The soil analysis was performed at the Department of Soil Science and Plant nutrition of the Namik Kemal University, Tekirdağ, Turkey as described by (Bouyoucos, 1951) The report showed that, the soil texture of the location was of loamy clayed with pH of 7.78 (Sağlam, 2012), EC of 866 dS/m, total nitrogen of 0.102% (Sağlam, 2012), extractable phosphorus content of 10.83 mg/kg, the extractable potassium content of 209.6 mg/kg and organic matter of 1.37% (Greweling ve Peech, 1960).

Statistical analysis

Each experimental treatment used 48 explants divided into equally distributed 12 replicates; each containing 4 explants. The data was analysed by comparing means using SPSS 24 program for Windows. The significance differences among means were determined by Duncan's multiple range test. The percentage of data obtained from the experiments was subjected to arcsine transformation before statistical analysis (Snedecor and Cochran, 1976).

Results

Static liquid culture

The single bulb scales transferred to stationary liquid MS medium; containing any concentration of TDZ used singly or in combination with any concentration of NAA did not induce bulblets. The scales began to deteriorate after 9 - 10 hours of culture. Bursting of tissue cells was visible after 30 - 40 hours followed by induction of necrosis and slowly spreading lesions in ~ 50 hours. Consequently, complete death of cultured tissues was observed after 3-4 days depending on the concentration of phytohormones.

Seismomorphogenic treatments with shaken liquid culture

The single scales, transferred to seismomorphogenic shaking fluid culture medium having any concentration of TDZ used singly or in combination with any concentration of NAA likewise did not incite any bulblet on the explants. The changed morphophysiological portrayal of the explants showed thickened, increased but variable length and width under the influence of phytohormones without any mortality. The scales, induced variable number of bulb like

structures that did not grow in any case and started to show hyperhydricity following 10-17 days of culture, independent of the application of phytohormone combinations in the study these did not developed into bulblets.

Agar semi solidified culture medium

Effect of 0.18 μM TDZ + different concentrations of NAA on bulblet regeneration: MS medium having 0.18 μM TDZ with and without any concentration of NAA induced 100% bulblets induction (Table 2). It seemed as if solid medium induced better permissive stimulus on the explants for induction of somatic organogenic bulblet initials (Fig. 1b). Likewise, the 0.18 μM TDZ + NAA concentrations in the culture medium had significant role on the number of induced bulblets (Fig. 1c). These juvenile bulblet initials induced shape of bulblets under the influence of phytohormones concentrations and combinations. Induction of juvenile bulblet initials was observed after 3 - 8 days of culture after swelling of the explants and continued until 4 weeks. These bulblet initials converted to bulblet like structures after about 1.5-2 weeks both at meristematic ends and on the scales. 0.18 μM TDZ + low concentrations of 0.54 to 1.08 μM NAA in MS medium had stimulatory effects on bulblet induction. The treatments containing 0.18 μM TDZ + 0.54 and 1.08 μM NAA were early to induce bulblet to take a defined shape. Whereas, the bulblets induced on 0.18 μM TDZ + 2.16 to 3.124 μM NAA were late (37-40 days of culture) and slow to take shape. Correspondingly, these bulblets developed vegetative mature tissues and attained size after about 53 to 60 days. TDZ used singly induced less number of bulblets compared to the multiple bulblet formation in the presence of 0.18 μM TDZ + 0.54 or 1.18 μM NAA in the culture medium. Higher concentrations of NAA in the culture medium were also inhibitive to induce new bulblets. No induction was noted on all explants on cultures containing MS medium (control) that developed browning and later on necrosis and mortality.

Mean number of bulblets per explant on 0.18 μM TDZ remained 1.17. Using any concentration of NAA with 0.18 μM TDZ that had positive impact on induction of bulblets per explant that run from 1.67 to 4.25. 0.18 μM TDZ on concentrations \geq 2.16 μM NAA that were inhibiting and induced significantly reduced number of bulblets per explant. Maximum number of 4.20 bulblets per explant was noted on the MS medium that contained 0.18 μM TDZ + 1.08 μM NAA. A significant ($p < 0.05$) reduction in bulblet induction was noted on MS medium containing 0.18 μM TDZ + 2.16 and 3.24 μM NAA in the regeneration medium.

Table 1. Meteorological observations for the experimental area during 2014

Climatic parameters	Jan.	Feb.	Mar.	April	May	June	July	Aug.	Sept.	Oct.	Nov.	Dec.
Average temperature ($^{\circ}\text{C}$)	8.0	8.7	9.9	13.4	17.5	20.6	24.8	25.3	20.9	15.6	11.2	9.3
Average high temperature ($^{\circ}\text{C}$)	20.4	20.6	24.0	22.8	33.6	30.1	34.6	33.5	33.1	27.7	19.6	17.5
Average minimum temperature ($^{\circ}\text{C}$)	-2.5	1.4	1.8	4.9	11.0	10.5	17.1	16.1	13.6	5.3	1.3	-0.8
Precipitation	44.4	6.0	73.6	46.8	72.1	92.2	107.7	80.5	98.5	136.1	35.2	80.3
Average relative humidity (%)	87.4	83.2	81.6	83.3	80.3	77.9	73.0	74.5	78.1	79.8	85.2	89.1

Source: Turkish Republic General Directorate of Meteorology, Tekirdağ, Turkey

Number of bulblets per culture vessel run from 4.67 to 17.00 on MS medium containing 0.18 μM TDZ + all concentrations of NAA. Minimum and maximum of 4.67 and 17.00 bulblets per baby food culture jars was recorded on MS medium that contained 0.18 μM TDZ + 0.0 or 1.08 μM mg/L NAA in order. Each scale recultured for 4 weeks on MS medium with 0.18 μM TDZ + 0.0 or 1.08 μM mg/L NAA induced 3.8 + 0.4 bulblets which were repeatedly recultured to increase bulblet production. Altogether, this method enabled an estimated harvest of +35 bulblets from a single bulb scale in 12 months with 8 recultures.

The mean bulblet diameter (0.80 cm) on 0.18 μM TDZ did not shift too much in every treatment of regeneration in term of statistics; except that contained 0.18 μM TDZ + 2.16 μM NAA with minimum gain in bulblet diameter (0.23 cm). Irrespective of the concentration of TDZ and NAA in each of the experiment, the explants induced new bulblets that increased their diameter with each subculture. The bulblets diameters run from 0.23 cm to 0.83 cm using 0.18 μM TDZ + any concentration of NAA in MS medium. Least and greatest bulblets diameter of 0.23 cm to 0.83 cm was noted on MS medium that contained 0.18 μM TDZ + 1.08 μM NAA to 0.18 μM TDZ + 0.54 μM NAA in MS medium in order. It was also observed that first developing bulblets inhibited development of new emerging bulblets; therefore, these bulblets had to be pinched to overcome dominancy and give way to new bulblets for growth and development with leaves. All bulblets had solid, root initials and elongated appropriately. The best regeneration potential was noted on MS medium containing 0.18 μM TDZ + 1.08 M NAA. It was noted that a regular subculture of the newly induced bulblets resulted in a remarkable improvement in all parameters displayed in the tables. It was important to remove the plantlets from gelled media for better acclimatization outcomes with high recovery potential (Fig. 1h).

Effect of 0.27 μM TDZ and different concentrations of NAA on bulblets regeneration: Compared to the previous experiment, this study made use of 0.27 μM TDZ with and without 0.0, 0.54, 1.08, 2.16 and 3.24 μM NAA (Table 3). The experimental results showed 100% bulblet regeneration on all explants regardless of treatments except MS medium used as control treatment. However, the phytohormones concentrations and combinations had significant effect on changing number of bulblet induction and their diameters.

The explants began to thicken or swell 2-5 days after culture and were prone to induction of bulblet initials followed by their conversion to hard-differentiated mass of white to green new bulblets (Fig. 1d). These juvenile bulblet initials continued to grow until 3.5 - 4 weeks. MS medium

(Control treatment 1) failed to induce any bulblet initials, whilst, MS medium containing 0.27 μM TDZ that induced 1.75 bulblets/explant. MS medium with 0.27 μM TDZ + all concentrations of NAA used in this study induced 2.67-6.50 bulblets. No callus regeneration was noted in every case. Working along these lines, a proceeded cyclic regeneration of new bulblets was possible by six times reculturing of the explants that reduced in number with each reculturing of the explants.

It was noted that 0.27 μM TDZ + 0.54 and 1.08 μM NAA in MS medium had more positive influence on bulblet induction compared to MS medium using 0.27 μM TDZ singly (Fig. 1b). Other concentrations of NAA with 0.27 μM TDZ induced 2.67 to 6.50 bulblets per explant. These treatments were stimulating with rapid conversion rate from bulblet initials to bulblets taking 18-23 days to complete the process. These bulblets grew to vegetative maturity to attain solidness and size after about 48-53 days. Each of 2.16 μM +0.27 μM TDZ with 3.42 bulblets and 3.24 μM NAA+0.27 μM TDZ with 0.42 bulblets were inhibiting and had negative impact on number of induced bulblets per explant. These treatments created lethargically effects on the regenerated cells of the growing bulblets that showed slowness and late formation of bulblets in 28-31 days to take shape. These bulblets grew to vegetative maturity to gain size and solidness after about 54-61 days of culture.

Number of bulblets per culture vessel runs from 1.67 to 26.00 on MS medium containing 0.27 μM TDZ + all concentrations of NAA. Minimum and maximum number of bulblets were noted on MS medium that contained 0.27 μM TDZ +3.24 μM NAA and 0.27 μM TDZ + 1.08 μM NAA respectively. The bulblets diameter runs from 0.33 mm to 0.90 cm. The least bulblets diameter was noted on MS medium that contained 0.27 μM TDZ + 0.54 μM NAA. The highest bulblets diameter was noted on 0.27 μM TDZ + 1.08 μM NAA in MS medium followed very closely by the bulblet diameter (0.80 cm) gained on 0.27 μM TDZ. Exposure of the micropropagated bulblets to the 15 \pm 1 $^{\circ}\text{C}$ in the growth chamber for 30 days in the 24 hour dark photoperiod resulted in an increase of 3 - 4 mm in size of the bulblets. Rest of the regeneration treatments showed significantly reduced bulb diameters when compared. It was also observed that first developing bulblets inhibited development of nearby induced new bulblets; therefore, these bulblets had to be pinched to overcome dominancy to give way to adjacent bulblets for growth and development. All bulblets had solid, root initials and elongated appropriately (Fig. 1e).

Table 2. Effects of 0.18 μM TDZ + different concentrations of NAA on regeneration of bulblets after 4 weeks of culture from bulb scale of *L. candidum*

TDZ (μM)	NAA (μM)	Bulblet regeneration (%)	Number of bulblets/explant	Number of bulblets/ baby food culture jar	Bulblets size** (cm)
0.18	0.00	100.00	1.17 \pm 0.42 c	4.67 \pm 0.37c	0.80 \pm 0.26 a
0.18	0.54	100.00	3.00 \pm 0.42ab	12.00 \pm 0.24b	0.83 \pm 0.86 a
0.18	1.08	100.00	4.25 \pm 0.76 a	17.00 \pm 0.98a	0.77 \pm 0.37 a
0.18	2.16	100.00	1.67 \pm 0.46bc	6.67 \pm 0.99 c	0.23 \pm 0.14b
0.18	3.24	100.00	1.75 \pm 0.60 bc	7.00 \pm 1.07c	0.8 \pm 0.13a
Control (MS medium)		0.00	0.00	0.00	0.00

Means shown by different small letters in a single column are statistically different at 0.01 level of significance using Duncan multiple range test

Table 3. Effects of 0.27 μ M TDZ + different concentrations of NAA on regeneration of bulblets after 4 weeks of culture from bulb scale of *L. candidum*

TDZ (μ M)	NAA (μ M)	Bulblet regeneration (%)	Number of bulblets/explant	Number of bulblets/baby food culture jars	Bulblets size** (mm)
0.27	0.00	100.00	1.75 \pm 0.44 c	7.33 \pm 1.02c	0.80 \pm 0.11a
0.27	0.54	100.00	2.67 \pm 0.41c	6.67 \pm 1.02c	0.33 \pm 0.06b
0.27	1.08	100.00	6.50 \pm 0.97a	26.00 \pm 2.21a	0.90 \pm 0.08a
0.27	2.16	100.00	3.42 \pm 0.39 b	13.67 \pm 0.6b	0.43 \pm 0.06b
0.27	3.24	100.00	0.42 0.19c	1.67 \pm 0.28 d	0.47 \pm 0.06b
control (MS medium)		0.00	0.00	0.00	0.00

Means shown by different small letters in a single column are statistically different at 0.01 level of significance using Duncan multiple range test



Fig. 1. Bulblet regeneration of *L. candidum* using various combinations of TDZ+NAA: (a) culture of explants in baby jars; (b) induction of bulblet initials on single scale explant under the influence of plant growth regulators; (c) induction of bulblets on MS medium using 0.18 μ M TDZ + NAA concentrations; (d) and bulblets on MS medium using 0.27 μ M TDZ + NAA concentrations; (e) inhibition caused by that first developing bulblets on closely regenerated bulblets; (f) long (g) and short roots induced on 0.18 μ M TDZ + NAA and 0.27 μ M TDZ + NAA induced (1-1.6 cm long) bulblets; (h) acclimatized plants in the greenhouse; (i, j) acclimatized plants fourth year blooming in the field

Rooting and acclimatization

The randomly selected weight of ≥ 2.5 g and 0.5 cm diameter bulblets obtained from each of the two experiments were rooted on $\frac{1}{2} \times$ MS medium containing 2.45 μ M IBA showed variable rooting. Weight of ≤ 2.5 g and < 0.5 cm diameter bulblets was not optimum for rooting. Generally, the bulblets induced on 0.18 μ M TDZ + NAA induced 2 - 2.5 cm long roots and those regenerated on 0.27 μ M TDZ + NAA induced shorter (1-1.6 cm long) roots (Fig. 1f). It was noted a bulblet diameter of + 0.5 cm that had positive impact on rooting (Fig. 1e). Consequently, not all other sized bulblets were subjected to rooting. The induced roots were comparatively thick and brittle. It was important to remove jell adhering to the bulbs before taking them for better acclimatization outcomes.

These plants were very hard and faced no problem during acclimatization in the greenhouse when the humidity was gradually reduced from 80% to 45% in the greenhouse (Fig. 1i). All bulbs grown plants showed visible signs of growth. New thin and strong roots replaced the old roots. All (100%) plants showed acclimatization. Irrespective of the source of plants they induced flower buds and bloomed (Fig. 1h) with seed induction. These plants could be compared with naturally grown plants and were morphological similar. The plants were daintily watered once every two days. All of the plantlets bloomed after one year. All plants subjected to acclimatization survived under greenhouse. These plants are showing continuous flowering for four years in the fields (Fig. 1i-j). The present study reports the impact of Thidiazuron (TDZ) + NAA for building up bulblet regeneration system on single bulb scales used as explant under different conditions using stationary liquid culture, seismomorphogenesis culture medium and semi solid medium using agar gelled medium containing different concentrations of TDZ+NAA.

Stationary liquid culture medium

The single bulb scales in stationary liquid medium containing both phytohormones and sucrose was found not suitable for regeneration. A tissue culture vessel having 100 g fresh weight tissue in a litre is assumed to suffice oxygen respiration needs of plant tissues for 8.5 hours (Curtis, 2005). Unlike aquatic plants, the terrestrial plants lack aerenchyma, a spongy tissue that allows exchange of gases between the tissues and external environment. It is assumed that due to lack of inadequate transport of oxygen in static liquid culture, a difficulty was noted to maintain oxygen gradient and the thermodynamic equilibrium. Additionally, movement of high concentrated culture solution to cytoplasm resulted in bursting of cells (De la Vina *et al.*, 1999; Overmyer *et al.*, 2003). It is assumed that these kinetic limitations in morphogenesis resulted in difficulties to induce regeneration on the explants.

Seismomorphogenic treatments with shaken liquid culture

Despite the fact that seismomorphogenic or shake culture technique is simple; however, all plants are not appropriate for shake culture. Regardless of the treatments, hyperhydricity was noted on all explants. It is presumed that the hyperhydricity, would have resulted in variable inhibition to regenerate and multiply the explant cells (Park *et al.*, 2004; Lai *et al.*, 2005) There is a need to do more

detailed studies to find optimum approach meeting the needs of multiplication *in vitro* using seismomorphogenic treatments. These active restrictions in morphogenesis brought about troubles to utilize supplement elements and sugars in the way of culture medium and regeneration.

Semi solid agar containing medium

In vitro plant cell and tissue culture systems are often utilized by proficient plant propagators and nurseries to quickly develop supply of novel clones with attractive attributes, and generation of sound, disease free plant cultivars (Rout *et al.*, 2006; Ozel *et al.*, 2008a; Cardoso and Teixeira da Silva, 2013; Teixeira da Silva and Dobra 'nszki, 2013). The composition of plant growth regulators in the culture medium directs the organogenesis in plants (Parmaksiz and Khawar, 2006; Aasim *et al.*, 2009; Daneshvar-Royandazagh *et al.*, 2009). Similarly, (Burun *et al.*, 2013) also noted bulblet regeneration from *L. candidum* bulb scales on MS containing different doses and combinations of NAA, BA, Kn and 2iP. However, they noted maximum bulblet induction of 88.2% in MS supplemented with 0.1 mg/l NAA + 0.01 mg/l BA and the average number of bulblets per explant was 2.9. This study reports 100% bulblet induction.

Discussion

The present study reports the impact of Thidiazuron (TDZ) + NAA to build up *in vitro* bulblet regeneration system. It was uncovered that the productive *in vitro* bulblet regeneration could be easily acquired using TDZ singly or in combination of these phytohormones. Previous studies note prominent role of auxins or NAA used singly or in combination with other cytokinins like BA in promoting bulblet regeneration. The control treatments (MS medium) with no phytohormones failed to induce new bulblets and induced necrosis in confirmation to (Niimi *et al.*, 1995; Nakano *et al.*, 2000; Nhut *et al.*, 2001; Kumar *et al.*, 2001, 2005, 2007; Bakhshaie *et al.*, 2016). They had similar observations on *L. longiflorum* scale explants cultured on hormone less culture medium. This verifies necessity of using plant growth regulators for bulblet induction. Simmonds and Cumming (1976) observed that a blend of 2, 4-D and BAP in MS medium were the best to induce callus and bulblet regeneration on number of lily cultivars using NAA, 2,4-D or BA singly or in combinations. (Niimi and Onozawa, 1979) found that bulblet induction on *L. rubellum* Baker leaf explants was significantly influenced by MS medium containing 0.1 mg/l BA + 1.0 mg/l NAA. (Van Tuyl *et al.*, 1991) noted the effect of MS nutrient medium on regeneration on ovary ovule culture of a number of *Lilium* species, hybrids and cultivars. They reported MS medium containing 0.1 mg/l NAA supplemented with 5% sucrose to be the most efficient for regeneration. (Tribulato *et al.*, 1997) compared effects of different NAA, 2, 4-D, picloram or dicamba for regeneration on *L. longiflorum* cv. 'Snow Queen' and noted somatic embryos from cell suspension cultures in liquid MS medium containing 2 μ M dicamba (Godo *et al.*, 1998) 0.01 mg/l BA on agar solidified MS medium to be the best

concentration for maximum bulblet induction. Just like the results reported in the present study, they noted that addition of cytokinin (BA) used singly in liquid medium was inhibitory to induce new bulblets.

Lian *et al.* (2003) note that lily propagation through traditional means is slowly reducing due to high production costs and suggests that it can be replaced by micropropagation. It was noted that if the explants were recultured by subculturing after harvesting the bulblets; they induced new bulblets each time without any difficulty for about 6 times irrespective of the treatment and induced + 35 bulblets from a single bulb scale in 12 months. It is known that reculturing of explants help in reduction of costs. Reculturing of the explants has been reported for *Xanthosoma caracu* by Asokan *et al.* (1984); *Trichopus zeylanicus* by Krishnan *et al.* (1995) and *Gladiolus* by Ziv (1989) and *Eucalyptus* by Palta (1982). This is primarily used for sustained regeneration of new plantlets/bulblets, because it enables propagation through multiple induction of meristems on the explants by exploitation of old explants (tissues) and also save time to prepare the new explant. Therefore, this method may hold a significant promise for inducing true to type plants at a desirable rate (Krishnan *et al.*, 1995).

The bulblets were able to root only when they attained weight of ≥ 2.5 g and 0.5 cm diameter. All bulblets not meeting these criteria had no inclination to root proliferation in contradiction to (Arzate-Fernandez *et al.*, 1997; Azadi, 2007) on *L. ledebourii*; (Lian *et al.*, 2002; Bacchetta *et al.*, 2003) on *Lilium* oriental half breed, (Tanimoto and Matsubara, 1995; Nhut *et al.*, 2001; Nhut *et al.*, 2002; on *L. longiflorum*, Chang *et al.*, 2000 on *L. speciosum*, Jeong, 1996 on *L. concolor*, Yamagishi, 1998 on *L. japonicum*, Wawrosch *et al.*, 2001 on *L. nepalense*). This variation could be due to use of different plant species used in these studies having different needs for rooting.

Similarly, Akçal *et al.* (2016) noted effects of different incubation periods (10, 12, 14 weeks), incubation temperatures (10-15 °C, 20 -25 °C), auxin (IBA 100 ppm, IBA 200 ppm) doses and scale positions (outer, middle, inner, center) on bulblet formation were investigated. 1.47 unless per explant after 14 weeks with bulblet height (19.105 mm); bulblet weight (0.792 g) and bulblet diameter (13.282 mm) on outer scales at temperature of 10-15 °C. They induced rooting using 200 mg/l IBA shock (Saadon and Zaccai, 2013) also report bulblet regeneration using found that the best shoot regeneration could be achieved at 15 °C.

Acclimatization

Acclimatization and hardening of *L. candidum* is an important event as has been noted for many bulbous plants (Preece and Sutter, 1991; Paek and Murthy, 2002; Khawar *et al.*, 2005; Priyakumari and Sheela, 2005). *In vitro* strategies have turned out to be expanding imperative in the production of large amount of ornamental plants of good quality. This study is of enormous importance for regeneration of *L. candidum* making large-scale micropropagation feasible. Real issues of tissue culture plants is their encounter of transplantation shock (Ozel *et al.*, 2008b) that could be avoided significantly by covering

them with polythene envelopes or by maintaining high humidity after transplantation. Taking of these precautionary measures helped simple establishment of transplanted material in a brief timeframe.

Conclusions

Development of an effective regeneration and acclimatization system of *L. candidum* utilizing single scale explants gives a chance to utilize biotechnological techniques to multiply the plant with high potential outcomes for *L. candidum* propagation. The outcomes are extremely important and give strong data relating to commercial and agricultural proliferation. Expansion of this study intentionally may help in multiplication of this plant with unlimited consistent and safe Access throughout the year.

Acknowledgements

This study was supported by Namik Kemal University Scientific Research Projects (BAP Project No: NKUBAP.00.24.AR.13.03) and Department of Agricultural Biotechnology Namik Kemal University, Tekirdag, Turkey.

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