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Callus Induction and Micropropagation of *Lilium candidum* L. Using Stem Bulbils and Confirmation of Genetic Stability via SSR-PCR

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Abstract: Natural populations of *Lilium candidum* L. are remarkably affected by biotic and abiotic factors therefore there is a requirement to develop effective micropropagation protocol to provide mass production, multiplication and conservation of these plants. For this reason, this study was aimed to develop an efficient micropropagation method for multiple shoot production via somatic embryogenesis induced from *L. candidum* stem bulbils and also to determine the genetic stability of *in vitro* grown plants using SSR markers. The obtained results of this study are the first comprehensive reports including an investigation of genetic fidelity on somatic embryogenesis of *L. candidum*. After surface sterilization of bulbils, the calculated regeneration percentage of them was 89.5% and the callus induction was achieved using leaf segments of *in vitro* grown bulbils. The well formed somatic embryos were obtained from smooth whitish-yellow colored calli and these somatic embryos produced well formed healthy *L. candidum* seedlings with adventitious roots. All rooted seedlings were easily adapted to greenhouse conditions and the genetic stability of *in vitro* grown seedlings were determined by using SSR-PCR technique and it was calculated as 100%.

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1. INTRODUCTION

Lilium candidum being a member of Liliaceae family is a perennial herbaceous medicinal and aromatic plant [1]. Because of its attractive white flowers, aromatic and medicinal components, *L. candidum* has widely been cultivated in many countries such as in USA, Italy, Netherlands, Spain, Germany, France and Turkey [2]. *L. candidum* is a species adapted to the Mediterranean climate. The other *Lilium* species, which are spread in our country are distributed in areas under the influence of the Black Sea climate which is cooler and more rainy climate. Their natural populations are remarkably affected by biotic and abiotic factors such as anthropological pressure, diseases, pathogen attacks, carbon fuel pollution, dramatic climate changes, therefore there is a requirement to develop effective micropropagation protocol to provide mass production, multiplication and conservation of these plants [3].

Plant tissue culture techniques providing very useful approach for rapid propagation of plant species help to preserve especially the economically important and endangered species. *In vitro* propagation supports the proliferation of plant cells, tissues and organs by incubating

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them in an aseptic condition in liquid or on semi-solid nutrient medium [4,5]. Although the earliest studies on plant tissue culture date back to the early twentieth centuries, the main studies improved completely from 1970s onwards, as technological improvements began to be increased and theoretical limitations started to be overcome by expanding interest in usage of biotechnological techniques [6]. Until today many plant tissue culture techniques have been developed for improvement and breeding of different group plant species such as conifers [7], dicots [4] and monocots [8].

Somatic embryogenesis being one of the most important micropropagation tools has been applied throughout different types of *in vitro* systems for plant mass production. This tool serves also many advantages for *in vitro* propagation of true-to-type clones, rapid regeneration of genetic transformed and somatic hybridized plants and induction and selection of mutant types. Additionally, somatic embryogenesis plays an important role in key studies on totipotency and understanding of principle pathways of morphogenesis. Because of all these possible advantages of somatic embryogenesis, it has been tempting studies on investigation of *in vitro* conditions for somatic embryo induction of different plant species [9] and an excessive number of procedures for effective *in vitro* regeneration based on somatic embryogenesis have been developed for many economically important plant species [10].

The continuity of genetic stability during *in vitro* growing and subculturing periods is very important for clonal propagation of especially medicinal, aromatic, rare and endangered plant species [4]. It is important to maintain genetic stability in micropropagated cultures. Molecular markers are more stable and highly reproducible compared to the various morphological, cytological and protein markers used to detect variation in tissue cultivated plants. A molecular marker such as SSR, ISSR, RAPD, AFLP etc. is beneficially used in tissue culture studies to test the genetic stability of *in vitro* regenerates [11]. In all prokaryotes and eukaryotes, SSRs expressing sequences between 1 and 6 nucleotides on DNA are one of the preferred markers in genetic diversity studies due to their high mutation rate and consequently high polymorphism rate. They are an excellent source of polymorphism for eukaryotic genomes [12].

Although there are many published papers for method development on somatic embryogenesis of *Lilium* spp., [13-20], none of them reported genetic stability investigation after *in vitro* propagated *L. candidum* natural populations using SSR molecular marker techniques. The current study was carried out to improve efficient micropropagation method for multiple shoot propagation via somatic embryogenesis induced from *L. candidum* stem bulbils and also to determine the genetic stability of *in vitro* grown plants using SSR markers. The obtained results of this study are the first comprehensive reports including investigation of genetic fidelity on somatic embryogenesis of *L. candidum*.

2. MATERIAL and METHODS

2.1. Plant Materials

Plant samples belonging to natural populations of *L. candidum* L. were collected from Nif Mountain (İzmir, Turkey). The legal authorization letter for sample collection was obtained from Republic of Turkey Ministry of Agriculture and Forestry, document number 36178555-604.01.01/488551 and all collected samples were taxonomically identified by Dr. Hasan Yildirim and Dr. Ademi Pirhan.

2.2. *In vitro* Culture Establishment

The stem bulbils of *L. candidum* (Figure 1A-B) obtained from natural populations were sterilized according to surface sterilization protocol of Özüdoğru *et al*. [4]. After surface sterilization, the bulbils were transferred to semi solid Murashige and Skoog (MS) [21].

Medium supplemented with 4.44 µM 6-Benzylaminopurine (BAP), 20 g L^{-1} sucrose and 7 g L^{-1} agar. To enhance *in vitro* regeneration of *L. candidum* bulbils, surface sterilized bulbils were secondly transferred to Olive Medium (OM), [22]. Medium supplemented with 4.44 µM BAP, 20 g L^{-1} sucrose, 3.5 g L^{-1} agar and 1.5 g L^{-1} phytagel [23]. We used 29 stem bulbils for each experiment. For each experiment, approximately 30 leaf fragments were used as the explant source for callus induction. For callus induction, ~ 0.5×0.75 cm leaf fragments derived from *in vitro* regenerated bulbils (Figure 1C-D) were transferred to MS medium supplemented with 10.7 µM Naphthaleneacetic acid (NAA), 20 g L^{-1}sucrose and 7 g L^{-1} agar. Somatic embryos obtained from *in vitro* grown calli via OM medium were supplemented with 10.7 µM NAA, 20 g L^{-1} sucrose and 7 g L^{-1} agar (Figure 1E-F). It was determined that OM medium supplemented with 10.7 µM Naphthaleneacetic acid (NAA), 20 g L^{-1} sucrose and 7 g L^{-1} agar was more effective for somatic embryos, experiments were continued with this medium. All *in vitro* cultures were incubated at 25±2 °C, under a 8 hours dark / 16 hours light photoperiod, with light provided by cool daylight fluorescent lamps (50 $\mu mol^{-1}m^{-2}s^{-1}$).

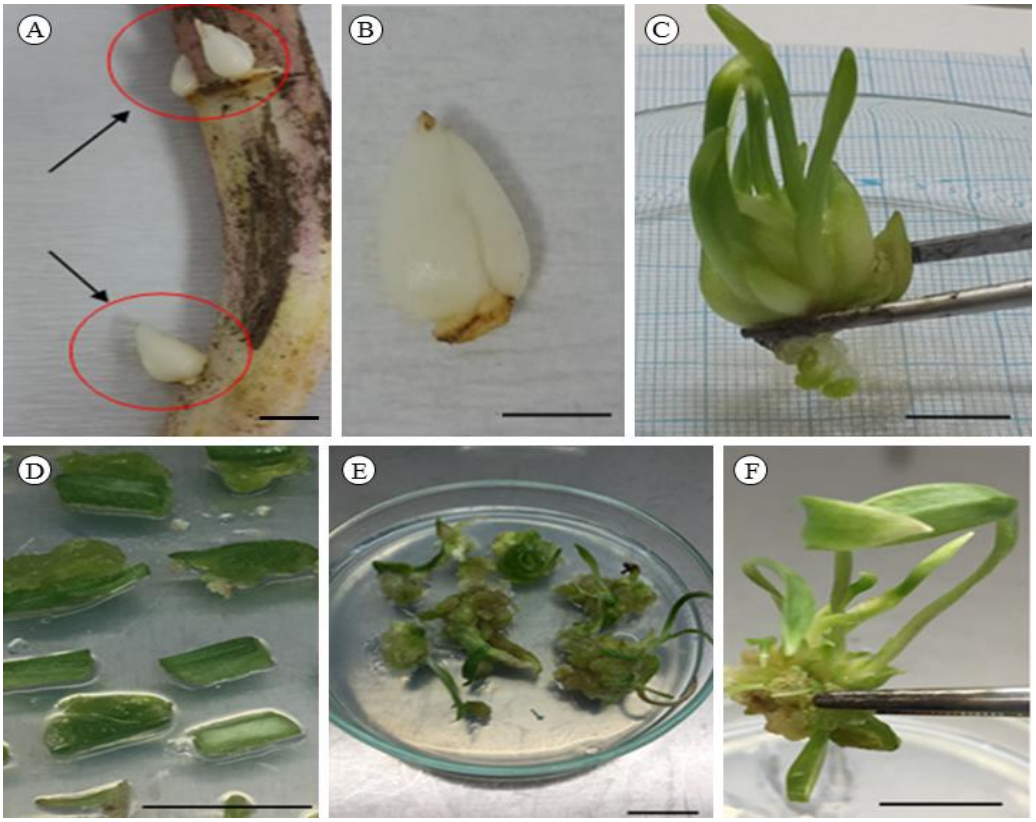

Figure 1. Morphological structure of *L. candidum* derived from native and *in vitro* grown plants (Bars 1cm) (**A**) Bulbils along the plant stem (**B**) One of stem bulbils used for *in vitro* culture initiation (**C**) *in vitro* regenerated bulbils (**D**) ~ 0,5×0,75 cm leaf fragments derived from *in vitro* regenerated bulbils (**E**) *in vitro* callus induction and somatic embryos (**F**) *in vitro* grown seedling obtained from callus.

2.3. Acclimatization to Greenhouse Conditions

Multiple rooted shoot clusters derived from *in vitro* grown somatic embryos of *L. candidum* (Figure 2A) were acclimatized under greenhouse conditions by transferring them into 100 mL plastic pots (Figure 2B) including nitrogen-rich peat and to gradually decrease the relative humidity of peat, the pots were closed with transparent pots and a hole was drilled on top of the transparent pots every day [4]. The plastic pots were irrigated with tap water for seven days, the transparent pots were removed after seven days and the plastic pots were transferred to greenhouse conditions (Figure 2C).

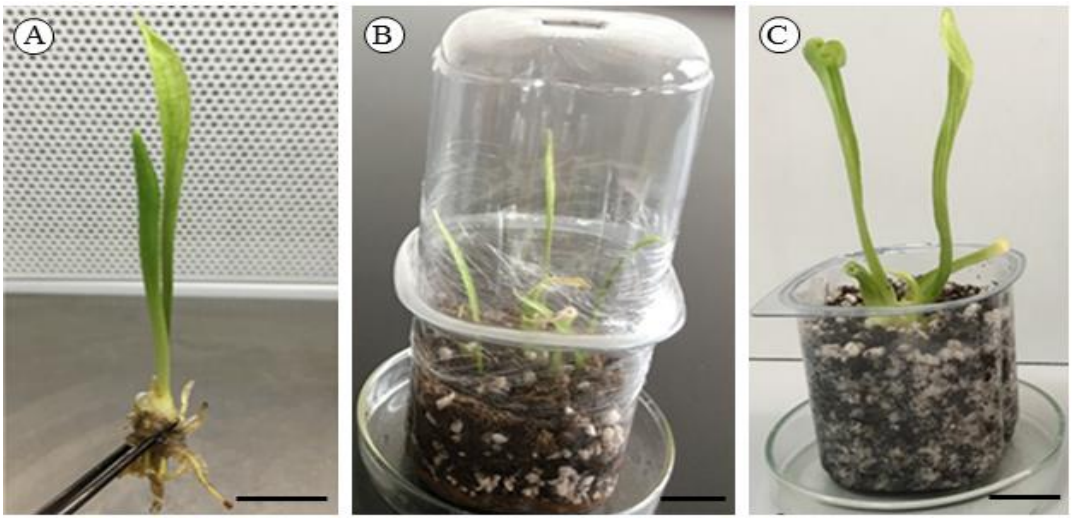

Figure 2. Acclimatization of *L. candidum in vitro* grown shoot clusters to greenhouse conditions (Bars 1 cm long) (**A**) Multiple rooted shoot cluster derived from *in vitro* grown somatic embryos of *L. candidum*, (**B**) Acclimatization under greenhouse conditions by transferring them into 100 mL plastic pots, (**C**) Transferring of acclimatized plants to green house conditions.

2.4. Data Collection and Statistical Analysis

In vitro regeneration of *L. candidum* bulbils, callus induction and somatic embryogenesis data were calculated as percentage values. All data were collected after four weeks incubation at standard culture conditions and all treatments were repeated at least three times. The statistical analysis of the non-parametric data was performed by means of the test for homogeneity rates, and the differences obtained by treatments were chosen using non-parametric statistical test [24]. Separate data were exposed to ANOVA, monitored by the least significant difference test at $P \leq 0.05$ to compare means. Data were analyzed by SPSS package program (IBM, version 21).

2.5. Determination of Genetic Stability

DNA isolation was performed from the mother plant, callus derived from *in vitro* regenerated bulbils, developing somatic embryogenesis, and acclimated seedlings (callus, somatic embryogenesis and acclimated seedlings were obtained after the third subculture) in order to confirm whether there was a variation. All samples were stored at -20 °C. SSR markers we previously determined as polymorphic were used in the naturally grown *L. candidum* genotypes.

2.6. DNA Isolation

DNA isolation was performed in all the above mentioned examples by modifying the method of Lodhi *et al*., [25]. After isolation, DNA samples were run on a 1% agarose gel to determine their purity and were visualized with a gel imaging system (MS, Major Science).

2.7. SSR Analysis

For SSR analysis, SSR primers to be used in the study were determined by referring to Du *et al*. [26]. Of these SSR primers, 12 pairs of them were identified as polymorphic in naturally grown *L. candidum* populations in Turkey (Table 1).

The total volume of PCR amplification reaction used in the study was 20 µL. Each PCR reaction consisted of 1×Taq PCR buffer, 0.2 mM dNTPs, 2 mM $MgCl_2$, 0.4 µM forward primer, 0.4 µM reverse primer, 0.2 units Go Taq Polymerase (Promega Go taq- M8295), 100 ng DNA and dH_2O. PCR amplification; The initial denaturation step was carried out at 94 °C for 5 minutes followed by 35 cycles of 1 minutes denaturation at 94 °C, 30 second annealing at (48-

55 °C) and 1 minutes extension at 72 °C with a final extension at 72 °C for 7 minutes. The PCR products obtained were run at a consistent voltage of 4.5 V/cm on a 4 % agarose (1.2% Biomax Basic Agarose and 2.8% Delta Micropor Agarose) gel in 1×TBE buffer for 3 hours by electrophoresis and bands were visualized by EtBr (Sigma-Aldrich®). Fragment size was determined using 100 bp ladder (ABM Cat: G016).

Table 1. SSR primers used for genetic stability determination in micropropagated *L. candidum* [26].

Primer no	Primer name	Repeat motif	Primer sequence (5'-3')
1	LS-ZJU07	$(AG)_6$	F:TGATCTCTGAGCTCCCCACT R:TGAGAATTGGATCAGGCGTT
2	LS-ZJU08	$(ACC)_7$	F:CATCAGCAACAACAAACCCA R:CTATGATTATAAGGCCGCCG
3	LS-ZJU09	$(CAG)_6$	F:AAGTCAGCAGCAACAGCAGA R:CAGGTAAAAATCCGCCAGAA
4	LS-ZJU11	$(GAG)_6$	F:TTCCAAGACCAGGACGACTC R:TTCCTGCCCAAATTGAACTC
5	LS-ZJU12	$(TC)_6$	F:CCATAGCTTCGTAGCTGCCT R:AAGTTGCCTAGAATGCCGAA
6	LS-ZJU13	$(GAC)_6$	F:GCTGTATAGCAGGACGGAGG R:TCGATTGTCTGCTTGACGAG
7	LS-ZJU16	$(CCT)_5$	F:GGCTCGCTCCTCTTCTCTCT R:GTCGTCCTAGCGGCATTAAG
8	LS-ZJU32	$(TTG)_5$	F:GTTTCCAACTGCGGATGTTT R:TGTTCAACTCCGTGCCACTA
9	LS-ZJU35	$(TGC)_5$	F:AAAAGCTCCAGCAAAAGCAG R:CTCCACCCTTGGATTTACGA
10	LS-ZJU40	$(TC)_6$	F:ATATCTTGACCCGCAGCATC R:AGCTCTGCAGGACGTTTGTT
11	LS-ZJU48	$(TGC)_6$	F:CTGCAGATGGAGATGCTGAA R:CCGTGAGAATGGTGTGAATG
12	LS-ZJU56	$(GA)_9$	F:TGAATGGGTAGGAGACGGAG R:TCCCCAATCAGACAATGTGA

3. RESULTS and DISCUSSION

3.1. Initation of *L. candidum in vitro* Cultures

After surface sterilization, *in vitro* culture initiation was provided by transferring *L. candidum* bulbils to MS initation medium described above, and obtained clean material percentage was calculated as 65.5%. The regeneration percentage of clean bulbils was calculated as 89.5% after four weeks incubation on OM regeneration medium described above. We used ~ 29 stem bulbis for each experiment, the clean material obtained showed regeneration of ~ 89.5%.

3.2. Induction of Calli and Somatic Embryogenesis

For each experiment, approximately 30 leaf fragments were used as the explant source for callus induction, The callus induction was achieved by using leaf fragments of *in vitro* regenerated *L. candidum* bulbils on MS callus induction medium described above and after two weeks incubation, the smooth whitish-yellow colored calli were successfully formed from widening and hardening leaf segments. Callus development was observed in each explant. The average callus induction rate per explant was calculated as 100%. After three weeks incubation, all cells of well formed calli produced healthy somatic embryos by transferring to OM somatic embryo producing medium described above. All of somatic embryos produced well formed healthy *L. candidum* seedlings and all seedlings have proper adventitious roots to adapt greenhouse conditions.

3.3. Determination of Genetic Stability

Micropropagation and callus culture experiments were carried out during the study to determine whether there were any somaclonal variations in the plants by the effect of nutrient media and growth regulators. For this purpose in the current study SSR markers were used and scanned for whether there was a genetic variation between the materials we produced by using micropropagation. The materials obtained from callus regeneration, somatic embryogenesis and acclimated plants were checked with SSR markers and the produced band profiles of these materials were found to be the same as the mother plant. The 12 pairs of primers produced 24 scorable bands (average: 2 bands/pairs of primer). As an example, SSR-PCR amplification of primer LS-ZJU 11 in DNAs of *L. candidum* mother plant, *in vitro* regenerated bulbils, *in vitro* grown somatic embryos and acclimatized plantles were visualized on agarose gel mixture is shown (Figure 3).

The plant tissue culture technologies having wide application area such as single *in vitro* cells, tissues and organs production, calli and suspensions in big-mass scale production, has become an important tool for plant biotechnology [27]. Plant tissue culture generally defining all procedures of *in vitro* cultivation, growth and maintenance of plant materials, has been developed and used for basic studies on cell differentiation, growth, division and fusion, plant physiology and biochemistry experiments, metabolic and genetic engineering, gene transformation, conservation of plant biodiversity [28]. In the current study, indirect somatic embryogenesis were achieved by using calli induced from *in vitro* grown *L. candidum* bulbils.

There are many studies on the selection of suitable nutrient media through the tissue culture of *Lilium.* These studies include studies on plant growth regulators [29-32], photoperiod application [33-37], explant size [29,30,33,36,38] and sugar concentration [29,30,33,36,38]. Altan and Bürün [39] reported that the MS medium supplemented with 0.1 mg L^{-1} NAA+ 0.01 mg L^{-1} BA, 30 g L^{-1}sugar and 8 g L^{-1} agar used was optimal in experiments for micropropagation of *L. candidum*. [39]. In the current study, it was determined that OM medium were supplemented with 10.7 µM NAA (2 g L^{-1}), 20 g L^{-1} sucrose and 7 g L^{-1} agar is effective for the development of somatic embryogenesis from callus obtained from *in vitro* regenerated bulbils.

Although callus induction was achieved by using MS medium, the somatic embryogenesis were obtained by using OM medium, both of media were supplemented with NAA. One of the main differences between the two media is that OM contains a different nitrogen salt [The OM medium contains a different NO_3^- [$Ca(NO_3)$, 2.54 mM] as nitrogen source and it also contains lower concentrations of other nitrate salts than the MS medium (NH_4NO_3, 5.15 mM; KNO_3, 6.09 mM)]. There have been many studies on potential benefits of different nitrogen sources such as NH_4^+ and NO_3^- and main purpose of these studies was to develop nutrient components of culture medium for different plant species. For example, the different concentrations of these forms of nitrogen in the nutrient media have produced very

positive responses on shoot regeneration [40], plant recovery efficiency in ovule cultures [41] and somatic embryo development [42]. In the current study, because of previous studies [3,43] in the literature, at first the MS medium was tested for *in vitro* propagation of *L. candidum*. However, in subsequent studies, OM [22] medium containing different concentrations of NH_4^+ and NO_3^- was reported to overcome possible growth problems after transfer to *in vitro* condition, accelerate the adaptation of the plant to the nutrient medium and provide better growth and it was obtained very positive results in comparison to MS [21] medium.

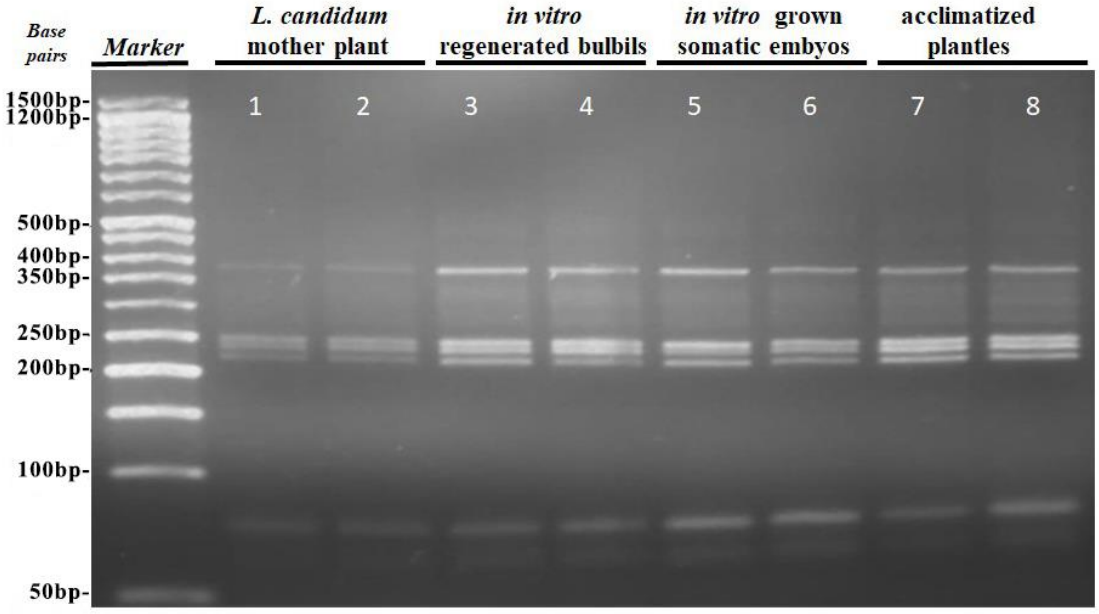

Figure 3. SSR-PCR amplification products obtained from DNAs of *L. candidum* mother plant (1, 2), *in vitro* regenerated bulbils (3, 4), *in vitro* grown somatic embryos (5, 6), and acclimatized plantles (7, 8) with SSR LS-ZJU11 [26] primers were visualized on agarose gel mixture (1.2% Biomax Basic Agarose and 2.8% Delta Micropor Agarose); M, 100 bp DNA ladder (ABM Cat: G016).

Plant cell and tissue culture have become a tool for the rapid reproduction of valuable species. Many plants can be produced with plant tissue culture by making continuous subculture under optimal conditions. However, in subculture studies performed by changing the growth environment (by accelerating or slowing down the growth), extending the changing intervals of the environment or increasing the number of subcultures have some risks. One of these risks is the emergence of somaclonal variations. Somaclonal variations are variations of genetic origin that occur between plants regenerated from somatic tissue originated callus, cell and protoplast cultures. These variations can be observed in plants as morphological, physiological and agricultural features. They are demonstrated by phenotypic, cytological and molecular level investigations [44]. Many reports have documented the assessment of genetic stability of micropropagated plants using SSR markers [12,45]. Liu and Yang [15] stated that 11 ISSR primers were used to determine the genetic stability of regenerated shoots in comparison to their mother plants. They reported that the genetic similarity between clonal samples and the mother plant was between 0.92 and 1.0. All 15 micropropagated materials and mother plants were grouped in a single master cluster with 92% similarity. They estimated the rate of somaclonal variation in plantlets to be 4.2%, emphasizing that direct shoot formation from explant regeneration indicates a safe method for the reproduction of "true–to-type" plants. Their results is acceptable for efficient mictopropagation, howewer, Asmita *et al*. [20] tested genetic stability using 10 SSR markers in twenty one *in vitro* regenerated plants. They produced a total of 273 bands from 10 SSR markers. The number of scorable bands for each primer ranged from

1 to 2. Among them, polymorphism information content was not recorded. Similar to our study, they stated that the banding profile of micropropagated plants is monomorphic and similar to the mother plant. Bi *et al*. [19] did not observe any polymorphism in embryo-like structure when analyzed with ISSR markers in five *Lilium* species and hybrids. Also, and no change in flow cytometry ploid level was observed. AFLP and ISSR markers have been used to detect genetic stability in direct shoot regenerants and ISSR markers showed no polymorphism but AFLP markers showed less than one percent [17]. Yadav *et al*. [16] used 6 RAPD markers and reported that there were no genetic variations in the regenerated micro bulbs. They stated that the results obtained through *in vitro* produced *Lilium* spp bulblets were clonally identical with mother plants. Varshney *et al*. [13] observed no change in progeny (randomly selected after four and 12 subcultures) with the RAPD marker.

4. CONCLUSION

In the current study, results of investigations based on SSR markers revealed that 12 pairs of primers produced amplified products with same monomorphic patterns of the mother plant, callus derived from *in vitro* regenerated bulbils, developing somatic embryogenesis, and acclimated material seedlings. It can be concluded that the results obtained by micropropagation protocol we employed here did not stimulate somaclonal variation in clones for these specific SSRs which were determined to be polymorphic markers in *L. candidium* genotype based on our previous studies.

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Declaration of Conflicting Interests and Ethics

The authors declare no conflict of interest. This research study complies with research publishing ethics. The scientific and legal responsibility for manuscripts published in IJSM belongs to the author(s).

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