Indian Journal of Agricultural Sciences **92** (7): 811–5, July 2022/Article
https://doi.org/10.56093/ijas.v92i7.81324

In vitro propagation and genetic fidelity evaluation in LA *Lilium*

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Received: 10 July 2018; Accepted: 25 March 2022

ABSTRACT

The present experiment was conducted at the Division of Floriculture and Landscaping, IARI, New Delhi, India during 2015–17 to develop a protocol for callus induction, PLB formation and plant regeneration from LA *Lilium* Brindisi using *in vitro* leaf segments, and to assess the genetic stability using SSR marker. Micropropagation of LA *Lilium* Brindisi led to compact calluses of dark brown to black colour. The *in vitro* regenerated leaves were inoculated at different concentration of 6-BAP and 2,4-D. Along with calluses, protocorm like bodies were also induced from the surface of cultured leaf segments, which further developed into shoots. MS medium fortified with 6-BAP (0.25 mg/l) and 2, 4-D (5 mg/l) recorded maximum callus formation. The mean number of shoot per callus clump ranged from 1.12 to 3.88, maximum number of shoots were recorded with 6-BAP (4 mg/l) and NAA (0.25 mg/l). Rooting ranged from 72–100% in IBA medium. Twenty regenerates were randomly selected for testing the fidelity. Out of 18 screened markers, only 10 produced clear and reproducible bands. A total of 244 bands were generated from 10 SSR primers in which seven primers were found polymorphic. Dendrogram generated by data analysis using Darwin 6 software package clearly indicated that the *in vitro* raised plants through leaf explant via callus phase were divided into three main clusters. The result of cluster analysis was supported by principal coordinate analysis (1/2 axis) where all the genotypes were distributed over different quadrangles. The total somaclonal variation was estimated to be 1.9% which indicated that even the plantlets raised through callus phase exhibited low frequency of somaclonal variation in case of LA hybrids of *Lilium*.

Keywords: Genetic fidelity, LA *Lilium*, Micropropagation, SSR marker

Lilium is an economically important monocot flower bulb grown as cut flower, pot plant and bedding plant worldwide. It belongs to the family Liliaceae and around 110 species are accepted under genus *Lilium* (GRIN 2018). Scale bulblets are conventionally used for multiplication at cheaper rates but inadequate availability of disease free quality planting material and slow multiplication rates limit the utilization. Tissue culture techniques provide an alternative with the production of large scale disease free quality planting material. In lily, plant regeneration has been reported from various explants such as leaves, stem nodes, filaments, pistils and scales (Yang *et al*. 2007, Mi and Liu 2008). *In vitro* adventitious plant regeneration from leaf explant has been reported by Bacchetta *et al*. (2003) in *Lilium longiflorum*, Kim *et al*. (2005) in *Lilium* Oriental hybrid, Xu *et al*. (2009) in *Lilium davidii* and Liu and Yang (2012) in *Lilium orientalis*. Till date, most of the protocols developed through different explants have mainly been established for Asiatic and Oriental lily. Systematic experiment is required on multiplication of LA hybrid lily under *in vitro* conditions and it needs to be standardized because of the species specific response of the various genotypes in *Lilium*.

For micropropagating ornamental crops, true-to-type and genetic stability of the plantlets are imperative for commercialization. For the regenerates produced by tissue culture, an important concern is their genetic fidelity with the mother plant (Martins *et al*. 2004, Modgil *et al*. 2005) because somaclonal variations can occur between regenerates produced from same explant during *in vitro* culture. To test genetic fidelity, molecular markers have been utilized in tissue culture research (Debnath 2010). Simple Sequence Repeats (SSR) marker is valuable and powerful tool used for analysis of genetic fidelity of *in vitro* regenerated plants. Due to highly polymorphic content and wide genome coverage, it is most suitable for assessment of genetic variation. Keeping this in view, the objective of the present study was to develop a protocol for callus induction, PLB formation and plant regeneration from LA *Lilium* "Brindisi" using *in vitro* leaf segments and to assess genetic stability using SSR marker.

MATERIALS AND METHODS

The experiment was conducted at the Central

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Tissue Culture Laboratory, Division of Floriculture and Landscaping, IARI, New Delhi during 2015–17; the molecular work was carried out at the ZTM & BPD unit, IARI, New Delhi during 2016–17.

Plant material: Lilium LA hybrid Brindisi leaves were *in vitro* regenerated to evaluate the key parameters affecting callus formation, PLB induction and direct regeneration. The explant source from the regenerated bulb scales was excised to 0.4–0.6 cm in length under laminar flow and was used for conducting the main experiment. The prepared explant was inoculated on petri dishes containing MS medium (Murashige and Skoog 1962), 3% sucrose, 0.8% agar and various concentration of 6-BAP, NAA and 2,4-D. The *p*H of the medium was adjusted to 5.78 before addition of agar and then autoclaved for 21 min at 121°C and 15 psi. Three replications per treatment with 20 explants per replication were initially incubated in the dark for 2 weeks and then transferred to 16 h photoperiod at 24±2°C. The data thus obtained were analyzed using DMRT.

DNA extraction, PCR amplification condition and SSR analysis: Young leaves (100 mg) of 20 *in vitro* derived plantlets were taken, these were selected randomly from a population of 250 *in vitro* stock shoot cultures in addition to mother plant. Extraction of the total genomic DNA was done using kit as described by Machery-Nagel (Duren, Germany). The quality and quantity of DNA was assessed by using Nano Drop Spectrophotometer. Initially, optimum PCR conditions for the different SSR markers were standardized using various concentration of template DNA (10, 20, 25, 50 ng/µl). The amplification was carried out using the kit (one PCR^{TM}, Helix Bioscience, New Delhi, India) in a total volume of 20 µl containing 25–30 ng of genomic DNA. Initially, 18 SSR markers were used for screening which were designed based on the previous report of *Lilium* SSR primers (Du *et al*. 2015). PCR amplification was performed. Initial denaturation at 95°C for 5 min followed by 35 cycles of 45 sec denaturation at 95°C, 45 sec annealing at 60°C and 45 sec extension at 72°C with a final extension at 72°C for 8 min. The amplified products were resolved on 3.5% agarose gel in TAE(1X) buffer stained with ethidium bromide and final picture was taken by Gel Documentation System (Alpha Innotech). The size of the amplicons resolved was marked by comparing with 100 bp ladder (Thermo Scientific). Scoring of bands was done based on the presence or absence in the gel photographs. Homology of bands was based upon their migration distance in the gel. Similarity between pair accession was calculated using Jaccard's similarity coefficient (J). The analysis was performed using Darwin 6 software package.

RESULTS AND DISCUSSION

Callus formation, protocorm like bodies (PLB) induction and plant regeneration: The *in vitro* leaves started showing response 20 days after inoculation in MS medium

Table 1 Effect of 6-BAP and 2,4-D on callus induction from *in vitro* leaves of LA lily

Treatment	Callus induction (%)	Days to callus induction
MS medium	0.00^{b} (0.00)*	0.00^{c}
MS + 6-BAP (0.25 mg/l) + 2, 4-D (1 mg/l)	0.00^{b} (0.00)*	0.00^{c}
MS + 6-BAP (0.25 mg/l) + 2, 4-D (2 mg/l)	0.00^{b} (0.00)*	0.00^{c}
MS + 6-BAP (0.25 mg/l) + 2, 4-D (3 mg/l)	1.35^{b} (6.68)*	33.76^{a}
MS + 6-BAP (0.25 mg/l) + 2, 4-D (4 mg/l)	13.36^{a} (21.43)*	28.92^{b}
MS + 6-BAP (0.25 mg/l) + 2, 4-D (5 mg/l)	15.40^{a} (23.11)*	29.16^{b}
MS + 6-BAP (0.50 mg/l) + 2, 4-D (1 mg/l)	0.00^{b} (0.00)*	0.00^{c}
MS + 6-BAP (0.50 mg/l) + 2, 4-D (2 mg/l)	0.00^{b} (0.00)*	0.00^{c}
MS + 6-BAP (0.50 mg/l) + 2, 4-D (3 mg/l)	0.44^{b} (3.80)*	30.28^{b}
MS + 6-BAP (0.50 mg/l) + 2, 4-D (4 mg/l)	12.80^{a} (20.96)*	30.12^{b}
MS + 6-BAP (0.50 mg/l) + 2, 4-D (5 mg/l)	14.76^{a} (22.59)*	30.04^{b}

Table 2 Effect of 6-BAP and NAA on shoot regeneration from calluses of *in vitro* leaves of LA lily

Treatment	Regeneration (%)	Days to shoot initiation	Number of shoots per callus clump
MS medium	7.96^{f} (16.34)*	20.16^{a}	1.12^{d}
MS + 6-BAP (1 mg/l) + NAA (0.25 mg/l)	37.56^{e} (37.79)*	18.96^{ab}	1.28^{d}
MS + 6-BAP (1 mg/l) + NAA (0.50 mg/l)	37.68^{e} (37.85)*	19.64^{a}	1.24^{d}
MS + 6-BAP (2 mg/l) + NAA (0.25 mg/l)	44.40^{cde} (41.78)*	17.52^{c}	2.24^{c}
MS + 6-BAP (2 mg/l) + NAA (0.50 mg/l)	42.32^{de} (40.58)*	17.56^{c}	2.20^{c}
MS + 6-BAP (3 mg/l) + NAA (0.25 mg/l)	54.32^{a} (47.49)*	17.04^{c}	3.52^{ab}
MS + 6-BAP (3 mg/l) + NAA (0.50 mg/l)	48.36^{abcd} (44.05)*	17.12^{c}	3.44^{ab}
MS + 6-BAP (4 mg/l) + NAA (0.25 mg/l)	52.12^{ab} (46.24)*	16.96^{c}	3.88^{a}
MS + 6-BAP (4 mg/l) + NAA (0.50 mg/l)	51.08^{abc} (45.59)*	17.04^{c}	3.44^{ab}
MS + 6-BAP (5 mg/l) + NAA (0.25 mg/l)	48.88^{abcd} (44.51)*	17.32^{c}	3.28^{ab}
MS + 6-BAP (5 mg/l) + NAA (0.50 mg/l)	46.32^{bcd} (42.88)*	17.80^{bc}	3.08^{b}

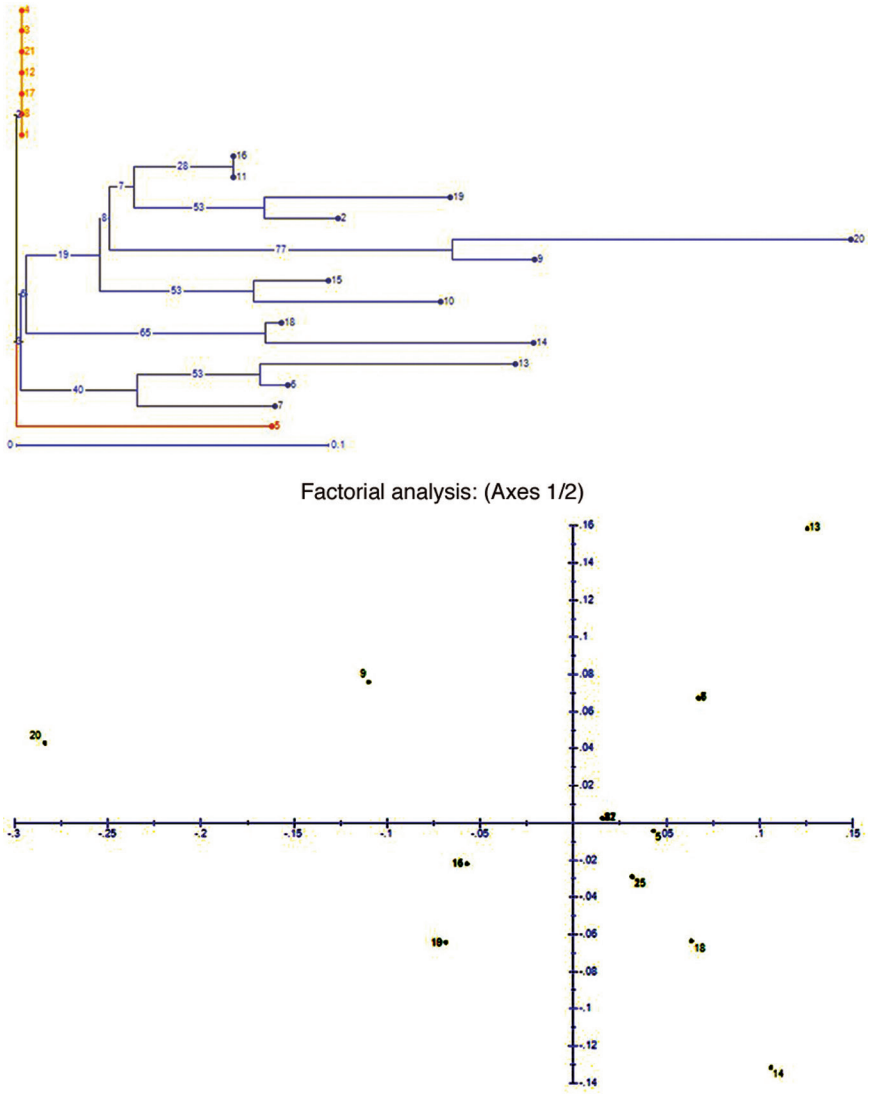

Fig 1 Dendrogram and Principle Coordinate Analysis obtained by UPGMA analysis with SSR markers for mother plant and *in vitro* raised plantlets from leaf explant derived callus in *Lilium* cv. Brindisi.

enriched with growth hormones (Table 1). Both calluses and PLBs were induced from the basal surface of leaves. Plants were regenerated both by direct (PLBs induction and direct regeneration) and indirect means (callus formation). The calluses formed were dark brown to black and had compact nature. Maximum callus formation was recorded in the MS medium fortified with 6-BAP (0.25 mg/l) and 2,4-D (5 mg/l), and minimum with 6-BAP (0.50 mg/l) + 2,4-D (3 mg/l) medium. Other leaf explant induced protocorm like bodies on the surface of leaves and proceeded to direct regeneration pathway forming plantlets. Leaves inoculated in the basal MS medium did not respond and finally died. Increase in the 6-BAP concentration decreased the percentage of callus formation. The calluses were further transferred to shoot regeneration medium for regeneration of shoot from callus (Table 2). Shoot regeneration frequency was found maximum when calluses were cultured with 6-BAP (3 mg/l) and NAA (0.25 mg/l) in MS medium while, mean number of shoots per clump ranged from 1.12 to 3.88. Use of leaf explant has been investigated earlier by Bacchetta *et al*. (2003), Kim *et al*. (2005), Xu *et al*. (2009) and Liu and Yang (2012) in Asiatic or Oriental lily. In the present experiment, leaf segments were first kept in dark for 2 weeks and then transferred to light condition for callus induction and plant regeneration where, formation of both calluses and protocorm like bodies was reported. Similar reports of keeping the explant in dark condition initially for 1–2 weeks was reported by Kim *et al*. (2005) and Liu and Yang (2012) in oriental lily. Compact and dark brown to black callus was obtained in the present experiment; Mori *et al*. (2005) recorded yellow and nodular callus on PIC containing medium. Callus formation has also been reported through different explant in different genotypes of *Lilium*. In the present experiment, formation of protocorm like bodies on the surface of leaf explant has been reported in LA *Lilium* hybrids. Formations of protocorm like bodies has been well recognized in orchids (Hong *et al*. 2008, Luo *et al*. 2008, Kaur *et al*. 2009, Mayer *et al*. 2010).

Rooting and hardening: Rooting was performed in the ½ strength MS medium supplied with different concentration of IBA (0.5 mg/l, 1.0 mg/l, 1.5 mg/l and 2.0 mg/l) after 4 sub-cultures at 4 weeks interval in shoot proliferation media. Rooting percentage ranged from 72–100%. The number of roots produced per micro-shoot ranged from 3.84 to 22.96. IBA (1.5 mg/l) supplemented in ½ MS medium was found to be the most effective. Rooted plantlets were hardened in medium containing sterilized peat and soil-rite mixture in glass jars covered with polypropylene caps where maximum survival percentage was recorded.

Assessment of genetic fidelity by SSR marker: The *in vitro* raised plants derived from the callus of leaf explants were subjected to SSR analysis for testing the genetic fidelity. Ten primers produced clear and reproducible bands out of 18 SSR primers. Total 12 bands were produced from 10 SSR primers with an average of 1.2 bands per primer. A total of 244 bands were generated in which seven primers were found polymorphic while others were monomorphic. The observed allelic size for each primer was almost in the same range as described by Du *et al*. (2015) in *Lilium*. Dendrogram generated using Darwin 6 software package (Fig 1) clearly indicated that *in vitro* raised plants were divided into three main clusters further divided into many sub-clusters. The

result of cluster analysis was further supported by principal coordinate analysis (1/2 axis) in which all the genotypes were distributed over different quadrangles (Fig 1).

Genetic stability in *Lilium* regenerated using meristematic based propagation system was reported earlier by Varshney *et al*. (2001) who did not detect variation among *in vitro* raised plants. Thereafter, Liu and Yang (2012) worked on ISSR marker in Oriental lily using leaves and scales as explant and observed 4.2% somaclonal variation in the plantlets developed by direct regeneration. Yin *et al*. (2013) used AFLP and ISSR markers to assess the genetic stability of plantlets regenerated from leaf segments of Oriental hybrid lily and recorded less than 1% polymorphism frequency. In all the above studies done on *Lilium*, no callus phase was involved in the plant regeneration, instead it was derived through adventitious shoot bud formation on the leaf explant. Genetic fidelity has also been detected in *Clivia miniata* (Wang *et al*. 2012), rose (Senapati *et al*. 2012), Asiatic hybrid lily (Yadav *et al*. 2013). During *in vitro* propagation, the genetic variation mainly depends upon the source of explants and the mode of regeneration (Goto *et al*. 1998). Plantlets derived through callus often exhibit the tendency of somaclonal variation when compared to embryogenic tissue (Rani and Raina 2000). So, it is essential to check the genetic stability of the plantlets derived via callus phase.

Being species specific, SSR marker allows best detection of the variability among the regenerates as compared to other PCR based markers. SSR markers are simple, consistent and highly efficient in evaluating the genetic diversity and phylogenetic studies (Kalia *et al*. 2011, Du *et al*. 2015). In the present study of *in vitro* regeneration through leaf explant, somaclonal variation was detected by DNA based SSR primers. It recorded only 1.9% somaclonal variation which is permissible for the large scale multiplication by micropropagation techniques. Hence, leaf explant can be successfully used for commercial multiplication of LA hybrids of *Lilium* with minimum risk of genetic variability.

ACKNOWLEDGEMENTS

The authors are grateful to University Grants Commission, Ministry of Human Resource Development, and Government of India for the financial assistance.

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