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Micropropagation and Genetic Fidelity of *in vitro* Grown Plantlets of *Begonia malabarica* Lam.

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Abstract: Begonias are ornamental indoor plants and also cultivated for landscaping. *Begonia malabarica* Lam., found naturally in the hilly regions of South India and Sri Lanka called by the tribals as Narayana Sanjeevi i.e., possess miraculous therapeutic properties. The present study targets *in vitro* culture, field establishment and evaluation of its genetic uniformity using anatomical, molecular markers followed by callus induction and establishment of cell suspension. Explants such as shoot tip and leaves from *in vitro* germinated seeds were cultured on Murashige-Skoog medium. Maximum callus induction was obtained in Murashige-Skoog medium with 0.3 mg L^{-1} 2, 4-D + 0.5 mg L^{-1} BAP i.e., 90.8 ± 2.8 % with 2.5 ±0.1 g callus growth. *In vitro* suspension of cells and their growth rate were also analyzed. RAPD and ISSR experiments were carried to confirm the genetic fidelity among *in vitro* and *in vivo* plants. 12 RAPD and 7 ISSR primers raised 119 amplicons. Genomic DNA amplification showed similarity between the *in vivo* and the *in vitro* generated plantlets. The overall results revealed the uniformity of the *in vitro* raised *B. malabarica* plantlets in terms of histoanatomical genetical features and substantiated the assumption that *in vitro* micropropagation is the secure mode for mass propagation of true to type plants without any somaclonal variations.

Keywords: *In vitro, Begonia malabarica*, genetic fidelity, callus, molecular markers

INTRODUCTION

Globally, the commercial mass production of indoor plants is a growing interest among horticulturist. There is a high demand for ornamental plants in both domestic and international markets and was significantly increased over the last two decades (Jain 2002). The plants such as *Begonia, Ficus, Anthurium, Chrysanthemum, Rosa, Saintpaulia,* and *Spathiphyllum* were being micropropagated in large scale in the nurseries of developed countries. Similarly, many indoor horticultural species were mass micropropagated via plant tissue culture programmes. Chebet *et al.* (2003) analyzed the application of *in vitro* culture with different growth regulators to

enhance the quality and multiplication of plantlets from the shoot tip explants of *Dendranthema morifolium*.

Further, biopharming process was formulated to boost economically important secondary metabolites. Such systems may solve many of the intrinsic problems faced, during industrial scale-up, on the extraction of these compounds from plants cultivated in the open field or collected from wild habitats. *Begonia malabarica* Lam. of Begoniaceae is used as coloured juice to provide vitality and stamina as reported by the Malasar and Malai Malasar tribes of Pollachi (Mohan *et al.* 2008). Five or six fresh leaves or five to ten grams of powder prepared from the shade dried aerial part of the Begonia malabarica is taken as an astringent by the Palliyar tribals of Saduragiri hills, Western Ghats, Tamil Nadu. Kanikkar tribals of Agasthiarmalai Biosphere Reserve, Tamil Nadu, used the fresh leaf juice of Begonia malabarica with salt to treat giddiness.The paste prepared from 10 gram of fresh leaves Begonia malabarica is applied externally once in a day for one week to treat foot sores by Kanikkar tribals of Agasthiarmalai Biosphere Reserve, Tamil Nadu (Mohan *et al.* 2008).Begonias are ornamental perennial herbs with soft, succulent stems with variable flower and leaf colour like white, pink, red, orange, or yellow. Family Begoniaceae comprises of 5 genera and 920 wild species. Begonia cultivars are a taxonomic puzzle due to their similarity of characters with their wild related species. Horticulture researchers categorized begonias into 8 groups: cane-like begonias, rex-cultorum begonias, rhizomatous begonias, begonia semperflorens, shrub-like begonias, thick-stemmed begonias, trailing or scandent begonias and tuberous begonias.

Floriculture industry estimates approximately billion of plantlets to meet the indoor garden designing (Marques and Caixeta 2003). Similarly, the food yielding hybrid species production varies between the countries. Chen *et al.* (2005) reviewed the *in vitro* growth parameters for optimal multiplication of ornamental plants. In global scenario, ornamental species generate more economy than food yielding and other similar categories.

Micropropagation of *Begonia* species is very tedious due to the hairy and succulent nature of the plant. Murashige-Skoog medium was the most effective one for micropropagation of the many ornamental plant species (Bouman and De Klerk 2001). Commonly, regeneration and differentiation of adventitious shoots is induced by cytokines. Further, if the pathway is established by hormonal combinations and then the regenerative tissue will follow the morphogenetic developments even in the absence of growth regulators. This strategy was successful to initiate *in vitro* cultures of *Begonia*, from different explants of the same plant material (Ab Aziz Rosilah *et al.* 2014).This method was also efficient for *in vitro* culture of other demanded ornamental species. In this scenario, the present study was aimed at *in vitro* mass multiplication of *Begonia malabarica* Lam. its establishment of callus, cell suspension and also validation of genetic true type by molecular and histological markers. The present study provides a standardized protocol for micropropagation and establishment of cell suspension, thereby giving an initial step for the cost effective production of anthocyanin from friable callus of *Begonia malabarica.*

MATERIAL AND METHODS

Plant material and initiation of aseptic cultures

The fresh healthy plants (60 cm length) of *Begonia malabarica* were collected from the wild habitat of Kallar region of Ponmudi Hills, Thiruvananthapuram, Kerala and the identified voucher specimen was deposited in the herbarium of University College, Trivandrum (Herbarium voucher specimen Number: UCB 1207).

Initiation of aseptic cultures using seed germination

Various surface sterilization protocols were adapted using sterilants such as mercuric chloride (0.01-0.1%) for 3-8 min, sodium hypochlorite (5- 15%) for 5-10 min, calcium chloride (10-15%) for 10 - 20 min for all the excised explants. Invariably, Tween 20, the polyethoxylated sorbitan ester, an emulsifying, hydrophilic and solubilising agent was also added with all the treatments. The surface sterilized seeds were inoculated in different media with varying concentrations of agar, sucrose and hormones. Medium used for the culture of seeds included : a) tap water + agar without sucrose (in which no other nutrients were added so that the germination of seeds occurs due the absorption of nutrients present in the tap water alone); b)basal medium; c) ½ Murashige-Skoog medium with different percentage of agar (0.2-0.7%) as solidifying agent along with different concentrations of sucrose (0.5-3%) were trialed. Sterilized seeds were also inoculated on MS medium fortified with different combinations and concentrations of Benzylaminopurine (BAP) (0.1-0.5 mg L^{-1}) and Naphthaleneacetic acid (NAA) (0.01-0.05 mg L^{-1}).

In vitro culture protocol

In vitro developed young *B. malabarica* plantlets produced after repeated sub cultures were employed as explants. The sterilized leaf and nodal explants were inoculated in MS medium fortified with different doses of 2,4-Dichlorophenoxyacetic acid (2, 4-D) (0.1- 1 mg L^{-1}) alone or in combination with BAP (0.5 mg L^{-1}) for the callus induction.

In vitro leaf segments were excised into approximately 1.0 cm^2 and cultured onto MS medium trialed with different concentrations of 2, 4-D (0.1-1.0 mg L^{-1}) with or without combination of 0.1-1.0 mg L^{-1} BAP for embryogenic callus induction with 12 replications. The *in vitro* cultures were kept in light/dark (16h light period/ 8 h dark period) continuous photoperiod with temperature 25 ± 2 ^{0}C. Friable embryogenic calli were identified and was subsequently cultured within 2 weeks in the fresh MS media to induce the formation of somatic embryos from vegetative tissues. The developments of somatic embryos were viewed using a compound microscope regularly in one week interval.

Plantlet regeneration

Somatic embryos developed from the leaf explants showed various stages like globular shaped, heart shaped and torpedo structured. The embryos were transferred individually into half MS basal media for plant regeneration. The cultures were incubated at 25 ± 2 ^{0}C, 16-h photoperiod with approximately 45% relative humidity.

Establishment of cell suspension culture

The friable callus obtained in MS medium supplemented with 2,4-D was used for the establishment of cell suspension. Friable callus (ie., 1 g callus in a 250 mL flask) were subcultured in 40 mL of liquid MS culture medium containing 2,4-D (0.1 mg L^{-1}), NAA (0.5 mg L^{-1}), and 6-BA (0.5 mg L^{-1}) combinations every week until the cells displayed uniform and optimal biomass. The physical parameters were 25 ± 1°C on a rotary shaker with a speed of 80 rotations per minute (rpm) under 16 h illuminations with 60 µmol m^{-2} s^{-1}. The medium was sterilized at 121 °C for 15 min. After repeated culturing for 20 days the suspension of cells were isolated through a Buchner funnel, gently cleaned with sterilized deionized distilled water to remove contaminations for thrice. Then the weighed fresh cells from the subculture (14^{th} day) (FW) were dried at 50 °C to obtain dry weight (DW). Cell growth was measured based on the FW and DW.

Anatomical studies

For the anatomical study, fresh *in vivo* and *in vitro Begonia* leaves were selected. Transverse section of leaves were taken, stained with Safranin (1% w/v) mounted on a glass slide with glycerol: water (1:1) and finally the sections were viewed through a compound microscope.

Isolation of DNA and evaluation of genetic polymorphism

DNA was isolated from fresh leaves of 120 days old cells of *in vitro* plantlets kept at green house following the protocol of Doyle and Doyle (1987). DNA aliquot of 3 µl was evaluated in terms of the quantity and quality of DNA. The yield of DNA ranged from 40 to 150 ng. RAPD primers (40) and ISSR primers (24) were used for DNA amplification with the *in vitro* and *in vivo* plantlets. Those primers yielded clear, visible and readable amplification were choosed for further DNA analysis (12 RAPD and 7 ISSR). Senapati *et al.* (2012) protocol was employed for the entire PCR programme. Amplification was performed in a PTC-100 thermal cycler programmed for a preliminary 2 min denaturation step at 94°C, followed by 40 cycles of denaturation at 94°C for 20 s, annealing at required temperature (depending on nucleotide sequence of RAPD and ISSR primer) for 30 s and extension at 72°C for 1 min, final extension at 72°C for 10 min. The amplified DNA fragments were subjected to electrophoresis in 0.8% agarose gel along with standard molecular ladder and viewed under UV light. The amplicon sizes were recorded using the GENSTAT cluster analysis software. Entire PCR experiments were carried out thrice to confirm the reproducible amplicons produced.

Statistical analysis

The data were mean ± SD from 12 trials. Statistical significance of difference between various treatments were analyzed by Students 't' test followed by one-way analysis of variance (ANOVA). Significance level was recorded at $p< 0.05$. Entire analysis was carried using SPSS (Version 9, SPSS Inc; USA).

RESULTS AND DISCUSSION

Seed germination and shoot proliferation

In vitro germination of seeds were achieved successfully on half strength MS medium with 0.5% agar + 0.5% sucrose without any plant growth hormones (96% of seeds cultured in half strength MS medium germinated within 20 days) (Fig. 1 a, b). The values are statistically significant with F value 87.88**. Meanwhile, MS medium with BAP+NAA yielded significant germination rate with vigorous growth. Further, the effectiveness of BAP and their combinations with NAA were evaluated for shoot multiplication from the explants shoot tip of the *in vitro* germinated seedlings. Varied morphometric responses were visualized with various concentrations and combinations of growth hormones. Interestingly, shoot induction was completely failed in the MS medium without growth hormones. However, BAP 0.4 mg/L + 0.01 mg/L NAA proved to be the ideal combination (100% yield) and resulted a mean of 6.4 shoots with 5.7 ±0.49 cm shoot length (Table 1) . Kshirsagar *et al.* (2015) reported highest shoot proliferation (i.e., 6.8) in *Swertia lawii* in the MS medium fortified with BAP only. Meanwhile, Yang *et al.* (2013) reported that GA_3 retarded seed germination from 49 to 30%. Meanwhile, BAP and IBA at different doses in the MS medium significantly triggered the germination rate and shoot proliferation. Similarly, Chung *et al.* (2009) compared the *in vitro* germination of orchids and its mass propagation using different media and hormonal concentrations. MS media supplemented with BAP + NAA (0.5 and 0.5mg/L) was found to be optimal for germination of immature seeds of *Cymbidium aloifolium* and also showed vigorous seedling growth. Further, plant height, number of leaves, root length and biomass were showed variations with different sucrose concentrations in the medium (Table 2).

Hoque *et al.* (2007) analyzed the seed dormancy status of rice cultivars using different media with or without sugar (3%). The results revealed that sugar in the medium promoted germination effectively among the wild and cultivated rice genotypes.

Callus induction and cell suspension cultures

The *in vitro* raised plantlets were further used for initiating callus induction. Young leaf and nodal regions from the *in vitro* plantlets were used as explant sources for the induction of callus in the MS medium fortified with different doses of 2, 4-D alone (0.1-1.0 mg L^{-1}) or in combination with BAP (0.5 mg L^{-1}). 3% sucrose was mixed in the media containing 0.5% agar. Callusing was initiated within 16 days of inoculation and subsequently, calli were sub-cultured once in 2 weeks (Figs. 2 a, b, c, d, e & f).

The effects of various concentrations of 2, 4-D (0.1 – 0.5 mg L^{-1}) were evaluated for callus induction and its proliferation from leaf explants (Fig. 2b). Significant variations were observed in the fresh weight and percentage of induction of callus. Frequency of callus induction and the fresh weight of callus was 90.8 ± 2.8% and 2.5 ± 0.1g respectively in the MS medium complemented with 0.3 mg L^{-1} 2, 4-D + 0.5 mg L^{-1} BAP. The value was statistically significant with F value 135.78**. Increasing the concentration of 2, 4-D above 0.3 mg L^{-1}, resulted into browning and necrosis of the explants. The callus developed was whitish and friable in nature (Fig. 2b, c). Similarly, the nodal segments also revealed positive response of callus induction in the same concentration but with reduced percentage of yield ie., 80.20±0.78%. In most of the *in vitro* studies, 2, 4-D in the culture medium showed effective induction of calli in plants like *Gentiana straminea* and *Swertia mussotii* bookmark22(Balaraju *et al.* 2011; He *et al.* 2011). In the present

study, friable to compact with greenish – white coloured calli were obtained from leaf and nodal regions when cultured on MS medium fortified with 2, 4-D (0.1 mg L^{-1}) and also produced embryogenic cells. Interestingly, significant callus yield was noticed with leaf explants as compared to shoot. Optimal yield of compact yellowish green callus was attained on MS medium with 2, 4-D (0.5 mg L^{-1}) and BAP (0.5 mg L^{-1}) which was comparatively higher than MS medium complimented with 2,4-D alone. The value was statistically significant with F value for 2,4-D + BAP(0.5+0.5)- 128.29*. Callus yield ranged from 1.0 to 2.5 g fresh weight after 3 months of sub-culturing (Table 3a and b).

Somatic embryo induction

The friable callus with embryogenic nature was obtained from MS medium with 2, 4-D (0.1 mg L^{-1}) within two weeks. The embryogenic cells could be efficiently distinguished from the non-embryogenic cells i.e., embryonic cells were smaller, round-shaped with dense cytoplasm and active division while, the other cells were elongated, vacuolated, with poor rate of cell division. Further, these non embryonic cells were turned brown and eventually accounted to death in the course of culturing. The embryogenic cells divide further and developed into somatic embryos. Sub-culturing on half MS basal medium of well-developed somatic embryos with 3% sucrose in 0.5% agar resulted into rooting within 3-4 weeks (Figs. 2 d, e and f) and later developed in to new plantlets. Various developmental stages were noticed in the somatic embryos starting from clusters of globular embryos to torpedo through heart- shaped structures (Figs. 3 a,b,c). Most of the somatic embryos developed into plantlets within 3-4 weeks of sub-culturing.

Romocea (2011) trialed different combinations of phytohormones in the MS media for micropropagation of the Begonia cultivar 'ambassador'- white. Similarly, mass propagation of *B. pavonina* by somatic embryos from leaf explants was proved by Ab Aziz Rosilah *et al.* (2014). The effective combination was the MS medium with 1.0 and 2.0 mg $L^{-1}$of 2,4-D in combination with 1.0 mg $L^{-1}$of BAP. Mendi *et al.* (2009), commercially exploited ornamental *Begonias* by *in vitro* organogenesis using MS media fortified with BA+ NAA using various concentrations. Interestingly, 2,4-D, BA and NAA yielded the optimal regeneration than any other combinations.

Hardening of *in vitro* generated plantlets

Table 4 displays the shoot induction from leaf derived callus explants of the species. Best percentage of shoot induction (89.5 ± 1.2%) was noticed in the MS medium supplemented with 0.3 mg L^{-1} 2,4-D and 0.5 mg L^{-1} BAP. It displayed the highest number of mean shoots (11.5 shoots/callus) and shoot length (5.4 cm). The value was statistically significant with F value 89.69**. Meanwhile, for nodal explant the shoot induction and length were 9.6±1.01 and 4.8±0.37 respectively with the same hormonal combinations (Table 4). The value was statistically significant with F value 72.86**.

In the rhizogenesis part of the study, both NAA and IBA produced remarkable numbers of roots. Mean while, IAA produced significant root numbers than the other auxins(Table 5). Increased concentrations (over 2.5 mg L^{-1}) of IBA and NAA showed decreased number of roots. Maximum rooting %, number of roots and length were noticed with 1.5 mg L^{-1} IAA (62, 11, 2.8 respectively). However, the same hormones in the nodal shoots were 48, 6.4 and 2.3 respectively. The binded culture medium of well rooted plantlets from the optimal rooting media was washed

thoroughly and successfully transferred in to the paper pots containing a mixture of garden soil, sand and coco peat in 1:2:1 ratio (Fig. 4 and Table 6). Two month after transferring, 78% survival rate of the plantlets was noticed. In addition, the plantlets grew vigorously and exhibited true morphometric characters similar to the wild one and were transplanted at their natural habitats. Number of plants survived and its survival % were significantly higher with cocopeat : garden soil: sand ie., 48 and 78 with plantlets derived from leaf when compared with that of nodal derived plantlets (39 and 62% respectively) (Table 6). The values were statistically significant with F values 23.71** and 30.63** respectively. Chavan *et al.* (2013) established the hardening effectively with sandy condition and reported 92% of survival from *in vitro* generated plantlets of *Ceropegia panchganiensis*bookmark19. Thus, in the present study commercial *in vitro* multiplication and conservation of *B. malabarica* were established without harming the plants from their natural habitat.

Cell suspension culture

Plant cell suspension cultures are ideal source for the induction of shikimate pathway of secondary metabolite synthesis (Chattopadhyay *et al.* 2002). For example, Koul *et al.* (2009) reported enhancement in secondary metabolite synthesis from *Swertia chirata* via plant cell suspension cultures. However, no cell suspension study has been attempted with *B. malabarica*.

In the present investigation, cell suspension culture was established by culturing fresh calli in liquid MS media supplemented with definite combinations and doses of BAP, 2, 4-D and NAA (0.5, 0.1, 0.5 mg L^{-1} respectively). MS liquid medium supplemented with 2, 4-D (0.1 mg L^{-1}) and BAP (0.5 mg L^{-1}) showed well established suspension cultures i.e., suspensions without any aggregation or clumps of cells (Fig. 5). The *in vitro* suspension of cells from *B. malabarica* revealed optimal and steady biomass accumulation on day 14. This medium and growth hormone combinations was used for further growth kinetics studies. Cells in suspension exhibited optimal growth for first 14 days and thereafter reduction in fresh as well as dry weight of cells were observed (Table 7). The maximum fresh weight (2.9 g) and dry weight (0.64 g) was noticed at 14^{th} day of culture in liquid MS medium complimented with 2, 4-D (0.1 mg L^{-1}) and BAP (0.5mg L^{-1}).

Jain (1997) attempted mass multiplication and cell suspension cultures from *Begonia* and *Saintpaulia* cultivars. Romocea *et al.* (2010) initiated *in vitro* cultures of *Begonia erythrophylla* from axillary buds. Ab Aziz Rosilah *et al.* (2014) successfully completed somatic embryogenesis and *in vitro* multiple shooting of leaf explants of the endemic species viz., *Begonia pavonina*. Mendi *et al.* (2009) proved *Begonia* regeneration through organogenesis (direct). Rowe *et al.* (2016) standardized the effect of different doses of BAP and NAA in the cultivar *Begonia rex* 'Fedor'. Nada *et al.* (2011) proved multiple shoot induction and its *in vitro* establishment from petiole and leaf explants of *Begonia tuberhybrida*. Jelaska *et al.* (2001) established shoot organogenesis and its regeneration of *Begonia rex* using thidiazuron. de Klerk *et al.* (1990) evaluated the extent of micromorphological discrepancies in micropropagated plants of Begonia x hiemalis. All these data supports the present *in vitro* culture results of *B. malabarica*.

Leaf Anatomy

The anatomical characters of the leaves from *in vivo* and *in vitro B. malabarica* plants were examined using light microscopy. The gross leaf anatomy of *in vivo* and *in vitro* plants showed similar structural features such as i) an upper epidermis comprises a single layer of slightly tangentially elongated compactly packed cells, ensheathed by a thick cuticle ii) mesophyll was heterogenous and differentiated into palisade and spongy tissues iii) midrib was less prominent over the upper epidermis iv) palisade tissue was made up of minimum number of layers (2-3 layers) whereas, spongy tissue was well developed with 6-8 layers of cells v) mid rib was more conspicuous towards the abaxial side of the leaf than the adaxial vi) vascular system of the mid rib was composed of five vascular bundles vii) vascular bundles were collateral viii) lower epidermal cells were smaller as compared to the upper epidermis ix) in the region of mid rib both the epidermis cells were smaller and with almost rounded outline x) lower epidermal cells in paradermal sections, disclose hexagonal cells with straight walls (Fig. 6a).

Similarly, the leaf of *in vitro* raised *B.malabarica* also showed same morphological and anatomical features. However, the leaves produced from *in vitro* conditions were smaller in size and with membranous texture. Anatomically, vascular bundle number remains as single mass in the *in vitro* cultured plants compared to the *in vivo* wild plants (Fig. 6b).

Begonias seem to display a hydrophobic nature ie., possess convex shaped epidermal cells that prevent accumulation of water (Bhushan and Jung 2006). Anatomical features specified above in *Begonia malabarica* were substantiated by Lanovici (2010) and Lanovici 2011. However, *J. curcas* showed anatomical variations among the *in vitro* and *in vivo* plants and the differences noticed in the leaves was related with their responds to environments. Thickness of mesophyll region among *in vitro and in vivo* leaves of *B. malabarica* showed similar features among the plants. However, the plants in the greenhouse showed an additional palisade layer, which reflects the micro environmental influence on their cell division. Taha and Haron (2008) analyzed morphology and anatomy of flowers and leaves of *in vitro* and *in vivo* developed *Murraya paniculata* plantlets. Morphologically, they showed no differences among the flowers except their size i.e., *in vitro* plants were with smaller sized flowers and projecting stigmatic lobes. Variations were also noticed in the palisade layers and the nature of leaf epicuticle layer. Similarly, in the present study the leaves of tissue culture developed plantlets were reduced in size with thin texture. Thus, the overall anatomical features of the *in vivo* and *in vitro* plants displays similarity of anatomical features, therefore they are histoanatomically true types.

Genetic polymorphism

As the last part of the study genetic polymorphism of the *in vitro* raised plantlets of *B. malabarica* were compared with the *in vivo* wild plants analyzed using molecular markers. Initially, 40 RAPD and 27 ISSR primers were screened, among them 12 primers of RAPD and 7 primers of ISSR were subsequently used based on their performance to produce clear and reproducible amplicons. Meanwhile, the other primers were eliminated due to their non distinct unclear bands. 119 amplicons were visualized after three PCR replications. Devarumath *et al.* (2002) compared genetic similarity of *in vitro* plantlets of three superior tea clones of 2n and 3n nature employing the markers such as RAPD, ISSR and RFLP. Leroy *et al.* (2000) confirmed similarity between

somatic embryoids, regenerated plants and wild species of cauliflower by ISSR. Panda *et al.* (2007) evaluated genetic consistency of mass propagated plantlets of *Curcuma longa* by RAPD and also using cytophotometry experiments. Joshi *et al.* (2007) proved genetic true types of *in vitro* raised *Swertia chirayita* by ISSR markers. All these documents substantiate the genetic consistency of *in vitro* plants produced via tissue culture from *B. malabarica*. Moreover, all the RAPD primers produced scorable clear bands between 0.32 to 2.9 Kbp molecular mass. Amplification profile of the *in vitro* raised *B. malabarica* plantlets along with their mother plant has been displayed by RAPD primer gel profile using OPD-08 (Fig. 7) and ISSR primer IG-18 (Fig. 8). The total banding of PCR amplicons from *in vitro* plantlets along with their mother plant was seen to be monomorphic for the tested primers except a marginal number of weak bands. These weak bands were non consistent (polymorphic) during the repetition of PCR cycles. The mass and number of DNA bands produced by respective primers has been displayed in the Table 8. No clear polymorphic bands were noticed among the major bands pattern obtained, however some marginal unconspicous bands were seen in some of the primers though their occurrence was quite less during repetitions. The average genetic resemblence among the five month old *in vitro* cultured plantlets maintained in the field along with *in vivo* mother plants as explants was nearly 99.164%. So, the mean frequency of polymorphism (even considering the weak bands) accounts to less than 2%. Lack of polymorphism in RAPD and ISSR band pattern among the *in vitro* plantlets confirms a high level of genetic similarity and also suggests that this mass-propagation method is consistent to maintain genetic stability of *B. malabarica* multiplication for a commercial scale.

Sara *et al.* (2012) compared the similarity between the explant and *in vitro* raised *Begonia rex* in terms of morphometric parameters. Anusha *et al.* (2015) evaluated the genetic similarity of *in vitro* multiplied clones of *Celastrus paniculatus* by RAPD primers. Goyal *et al.* (2015) compared micro propagated plantlets of *Dendrocalamus strictus* using the molecular markers such as RAPD and ISSR. Kumaria *et al.* (2012) confirmed the similarity of *in vitro* raised *Begonia rubrovenia* var. meisneri, a rare, endemic ornamental species of Meghalaya. Raji *et al.* (2013) proved homogeneity of *in vitro* generated plants in terms of RAPD and morphometric methods in *Justicia tranquebariensis*. Senapati *et al.* (2012) showed genetic resemblance of *in vitro* raised plantlets of rose species via RAPD and ISSR molecular markers with their wild plants. Kshirsagar *et al.* (2015) showed regeneration of plants by *in vitro* culture and also established callus and suspension cultures of *Swertia lawii*. Shetty and Nareshchandra (2012) showed the uniformity of plantlets yielded via tissue culture protocols in *Withania somnifera* by genetic finger printing using RAPD protocol. All these experiments supports the present data of genetic fidelity of *in vitro* raised *B. malabarica* plantlets.

CONCLUSION

Commercially, micropropagation of horticrops is of high demand. International and national genetic engineering institutes design protocols for mass production of economically important plants. Biopharming is another application of production of phytochemicals with commercial importance. The present results suggest effective *in vitro* propagation of *Begonia malabarica* using 2, 4-D and BAP, (which stimulate callogenesis), and may be an opening for an effective

micropropagation protocol for *Begonia* species with a better regenerative capability and plant yield. Further, suspension culture yielded significant biomass and future demand for extraction of anthocyanin, a bioactive secondary metabolites which possess immense commercial significance.

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Table 1: MS medium with different hormonal combinations used for the germination of seeds with 0.5% agar (0.1 % Mercuric chloride pre-treated capsule)

Hormone combinations	Sucrose	Rate of germination	Growth	No. of days	No of multiple shoots
Tap water	-	70%	Slow	30	3
Basal medium	3%	50%	Slow	30	2
½ MS medium	0.5%	96%	Rapid	20	5
BAP+ NAA	3%				
0.1+0.01		80%	Slow	41	3
0.1+0.03		82%	Slow	40	3
0.1+0.05		84%	Slow	40	4
0.2+0.01		87%	Slow	38	4
0.2+0.03		86%	Moderate	37	3
0.2+0.05		88%	Moderate	38	4
0.3+0.01		90%	Moderate	34	3
0.3+0.03		89%	Moderate	34	2
0.3+0.05		90%	Moderate	34	2
0.4+0.01		99%	Rapid growth after germination	30	6
0.4+0.03		82%	Rapid	32	5
0.4+0.05		76%	Rapid	32	4
0.5+0.01		80%	Moderate	33	4
0.5+0.03		88%	Moderate	34	4
0.5+0.05		87%	Moderate	33	3
F	Tap water- 67.23*	Basal medium- 50.12	½ MS medium- 87.88**	BAP+ NAA- 99.56**	

Table 2: Mean values of growth parameters of *B. malabarica* plantlets on 1/2 MS containing different concentrations of sucrose (g/L)

Sucrose (S)	Plantlets height (cm)	Number of leaves	Root length (cm)	Biomass (g)
1	3.08 ± 0.45	3.63 ± 0.18	4.76 ± 0.86	0.013 ± 0.0
2	4.03 ± 0.34	6.14 ± 0.09	4.97 ± 1.21	0.023 ± 0.001
3	7.99 ± 0.18	11.47 ± 0.35	5.9 ± 0.50	0.061 ± 0.002
4	7.65 ± 0.71	10.03 ± 0.54	5.87 ± 0.23	0.057 ± 0.00
5	7.64 ± 0.10	9.14 ± 0.19	5.56 ± 0.56	0.06 ± 0.002
6	7.90 ± 0.53	5.68 ± 0.22	5.38 ± 0.36	0.059 ± 0.005
F	S 1g/L- 97.76 S 2g/L- 100.42*	S 3g/ L- 110.58** S 4g/L- 108.32**	S 5g/L-104.92*	S 6g/L-101.28

Table 3a: MS medium with different Hormonal combinations used for the callus induction from leaf explant

Sl. No	Hormone combinations mg/L	Fresh Weight of Callus (g)	Callus (%)	Callus percentage	Nature of callus	Callus colour
	2,4-D					
1	0.1	1	++	71.2±1.6	Friable embryogenic	Light yellowish
2	0.3	1.3	+++	80±0.9	Friable	Yellowish
3	0.5	-	-	0	Darkening of explant	-
4	0.9	-	-	0	Death of explant	-
5	1.0	-	-	0	Death of explant	-
F	2,4-D (0.1mg/L)- 116.23*			2,4-D (0.3mg/L)- 125.67**		
	2,4-D + BAP					
6	0.1+0.5	1.08	++	73.5±4.3	Slightly friable	Yellowish
7	0.3+0.5	2.5	+++	90.8±2.8	Compact	Yellowish
8	0.5+0.5	2.0	++++	79.2±5.1	Compact	Yellowish green
9	1.0+0.5	-	-	0	Death of the explant	-
F	2,4-D + BAP(0.1+0.5)-120.53		2,4-D + BAP(0.3+0.5)-135.78**		2,4-D + BAP(0.5+0.5)-128.29*	

Table 3b:MS medium with 2,4-D+BAP used for the callus induction from nodal explants

Sl. No	Hormone combinations	Fresh Weight of Callus (g)	Callus (%)	Callus percentage	Nature of callus	Callus colour
	2,4-D					
1	0.1	0.79	+	60±0.2	Friable embryogenic	Light yellowish
2	0.3	0.98	++	67±1.4	Friable	Yellowish
3	0.5	-	-	0	Darkening of explant	-
4	0.9	-	-	0	Death of explant	-
5	1.0	-	-	0	Death of explant	-
F	2,4-D (0.1mg/L)- 87.11*			2,4-D (0.3mg/L)- 91.23**		
	2,4-D + BAP					
6	0.1+0.5	0.92	++	69±0.86	Slightly friable	Yellowish
7	0.3+0.5	1.7	+++	80.2±2.4	Compact	Yellowish
8	0.5+0.5	0.94	++	65±0.95	Compact	Yellowish green
9	1.0+0.5	-	-	0	Death of the explant	-
F	2,4-D + BAP(0.1+0.5)-110.19*		2,4-D + BAP(0.3+0.5)-125.64**		2,4-D + BAP(0.5+0.5)-99.78	

Table 4: Effect of different concentrations of 2,4-D+BAP on number of shoots and length from leaf and nodal explants

Sl. No	Hormone combinations	Leaf (L)		Nodal (N)	
		No. of shoots/callus	Length (cm)	No. of shoots/callus	Length (cm)
	2,4-D				
1	0.1	3.5±0.11	1.9±0.1	2.6±0.05	1.3±0.01
2	0.3	6.9±0.25	2.6±0.3	5.9±0.08	4.2±0.05
3	0.5	-	-	-	-
4	0.9	-	-	-	-
5	1.0	-	-	-	-
F-L	2,4-D (0.1)-56.13*	2,4-D (0.3)-69.45**			
F-N	2,4-D (0.1)-50.67*	2,4-D (0.3)-61.66**			
	2,4-D + BAP				
6	0.1+0.5	5.8±0.31	3.9±0.22	4.7±0.09	3.1±0.02
7	0.3+0.5	11.5±0.87	5.4±0.17	9.6±1.01	4.8±0.37
8	0.5+0.5	10.0±1.02	4.5±0.13	8.3±0.35	4.0±0.43
9	1.0+0.5	-	-	-	-
F-L	2,4-D + BAP (0.1+0.5)-72.33	2,4-D + BAP (0.3+0.5)-89.69**		2,4-D + BAP (0.5+0.5)-83.57*	
F-N	2,4-D + BAP (0.1+0.5)-63.54	2,4-D + BAP (0.3+0.5)-72.86**		2,4-D + BAP (0.5+0.5)-67.50*	

Table 5: Effect of phytohormones on rooting %, number and length after sub-culturing of leaf and nodal derived shoots

Sl. No	Hormone combinations			Shoots rooted	No. of roots/shoot	Root length
	IAA	NAA	IBA			
1	0.5	-	-	32±1.5	3±0.04	1.9±0.05
2	1.0	-	-	43±0.71	7±0.35	2.3±0.02
3	1.5	-	-	62±0.28	11±0.01	2.8±0.04
4	2.0	-	-	40±1.3	6.1±0.26	2.5±0.07
5	-	0.5	-	40±0.2	6.4±0.18	1.4±0.03
	-	1.0		46±0.54	7.8±0.46	2.1±0.06
6	-	1.5	-	52±0.76	10±0.68	2.5±0.02
7	-	2.0	-	41±0.29	5.7±0.25	2.0±0.01
8	-	-	0.5	39±0.66	5.2±0.44	1.1±0.03
9	-	-	1.0	45±0.21	6.5±0.26	1.5±0.09
10	-	-	1.5	50±0.36	9±0.24	2.4±0.11
11	-	-	2.0	39±0.28	3.8±0.07	0.9±0.03
12	0.5	-	-	30±0.15	2±0.08	1.7±0.001
13	1.0	-	-	39±0.11	4±0.11	2.0±0.21
14	1.5	-	-	48±0.26	6.4±0.46	2.3±0.03
15	2.0	-	-	36±1.3	3.5±0.17	1.9±0.07
		0.5		32±1.9	2.4±0.25	1.8±0.06
		1.0		39±2.5	4.2±0.31	2.0±0.09
		1.5		43±3.6	5.7±0.39	2.1±0.1
		2.0		40±3.8	4.4±0.16	2.0±0.15
			0.5	28±4.5	1.7±0.25	1.5±0.08
			1.0	33±4.1	2.2±0.17	1.6±0.06
			1.5	39±3.0	4.2±0.67	1.9±0.14
			2.0	35±2.6	3.3±0.28	1.7±0.01
F	leaf-78.52**			Nodal-56.33*		

Table 6: Effect of substrate composition on survivability rate of plantlets derived from leaves and nodal explants

Hardening medium	Leaf (L)		Nodal (N)	
	No. of plants survived	Percentage	No. of plants survived	Percentage
Garden Soil (G)	17	30	14	26
Garden soil: Sand (1:1) (GS)	32	55	26	45
Coco peat: Garden soil(1:1) (CG)	39	61	32	50
Coco peat:Sand(1:1)(CS)	28	43	23	35
Coco peat: Garden soil :Sand(1:1:2) (CGS)	48	78	39	62
F-L G – 12.37	GS – 16.96	CG – 20.11*	CS – 15.53	CGS - 23.71**
F-N G – 15.98	GS –21.33	CG –27.74**	CS –16.31	CGS - 30.63**

Table 7: Fresh (FW) as well as dry weight (DW) of cells obtained in suspension culture for first 18 days

Days	FW (g)	DW (g)
2	0.89±0.21	0.10±0.01
4	1.12±0.19	0.22±0.05
6	1.45±0.35	0.30±0.02
8	1.7±0.02	0.34±0.04
10	2.0±0.15	0.4±0.01
12	2.2±0.86	0.43±0.001
14	2.9±0.28	0.64±0.02
16	2.9±0.16	0.64±0.11
18	2.7±0.92	0.58±0.06
F- FW	1735.196**	
F-DW	206.730**	

Table 8: RAPD and ISSR primers and their sequences with monomorphic band numbers of *in vitro* plantlets from *B. malabarica*

RAPD primers	Primer sequence	Monomorphic bands	ISSR primers	Primer sequence	Monomorphic bands
OPA-02	TGCCGAGCTG	4	IG-01	AGGGCTGGAGGAGGGC	7
OPA-04	AATCGGGCTG	4	IG-11	(AG)8C	7
OPD-02	GGACCCAACC	5	IG-16	(CT)8G	10
OPD-08	GTGTGCCCCA	4	IG-18	CAG (CA)7	9
OPD-11	AGCGCCATTG	6	IG-19	TGG(AC)7	8
OPD-18	GAGAGCCAAC	7	IG-22	(GACAC)2	8
OPD-20	ACCCGGTCAC	6	IG-23	(GA)8C	6
OPN-02	ACCAGGGGCA	4			
OPN-07	CAGCCCAGAG	8			
OPN-08	ACCTCAGCTC	5			
OPN-15	CAGCGACTGT	6			
OPN-16	AAGCGACCTG	5			
	Total	64			55

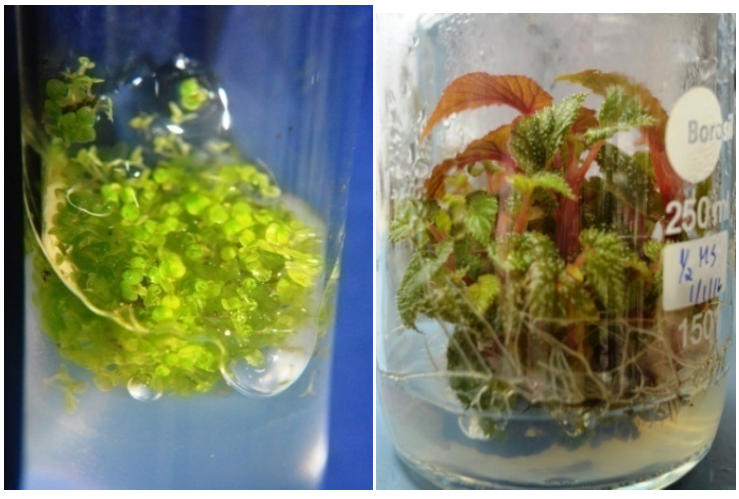
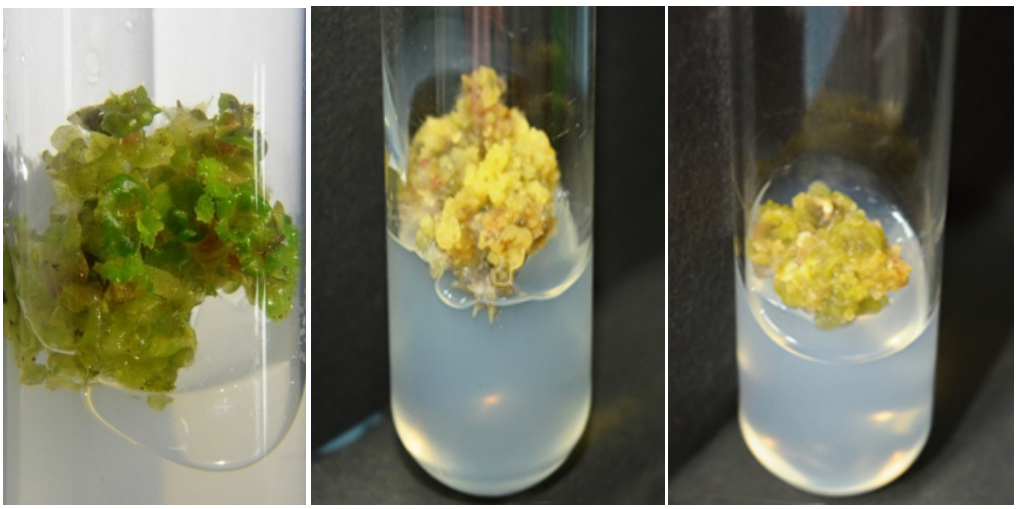
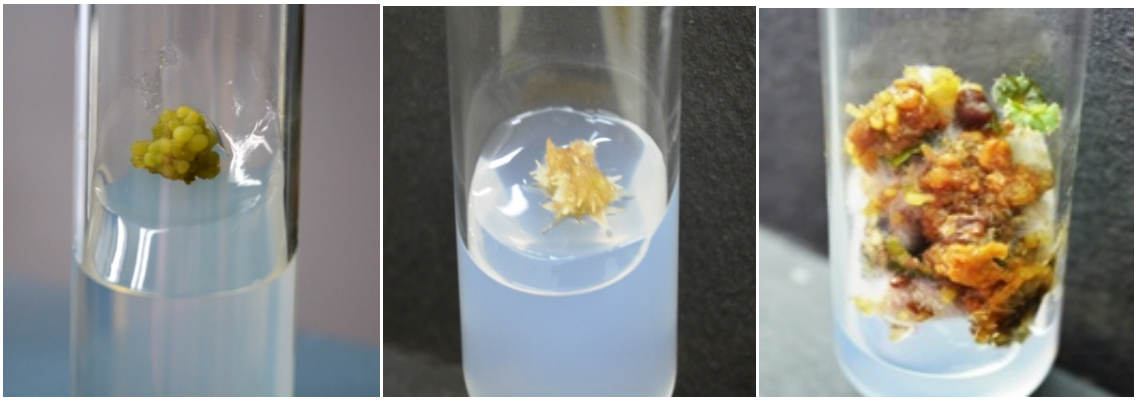


Figure 1. *In vitro* germinated seedlings on MS medium with 0.5% agar and sucrose

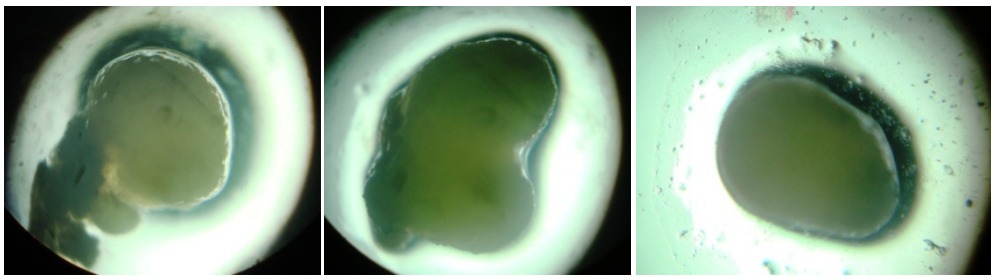


a b c



d e f

Figure 2. (a) MS medium supplemented with 2 mg L^{-1} BAP and 1mg L^{-1}NAA showing profuse growth and callus like out growth after 10 days of sub culturing; (b) Friable callus obtained on MS medium fortified with 2,4-D (0.1 mg L^{-1}) in leaf explants; (c) Good yield of compact yellowish green callus was obtained on MS medium with both 2,4-D and BAP (0.5mg L^{-1}); (d) Friable calli with embryogenic nature obtained on MS medium with 2,4-D (0.1 mg L^{-1}); (e) Sub cultured embryogenic calli on ½ MS medium producing root; (f)Regeneration of plant from embryogenic callus.



a b c

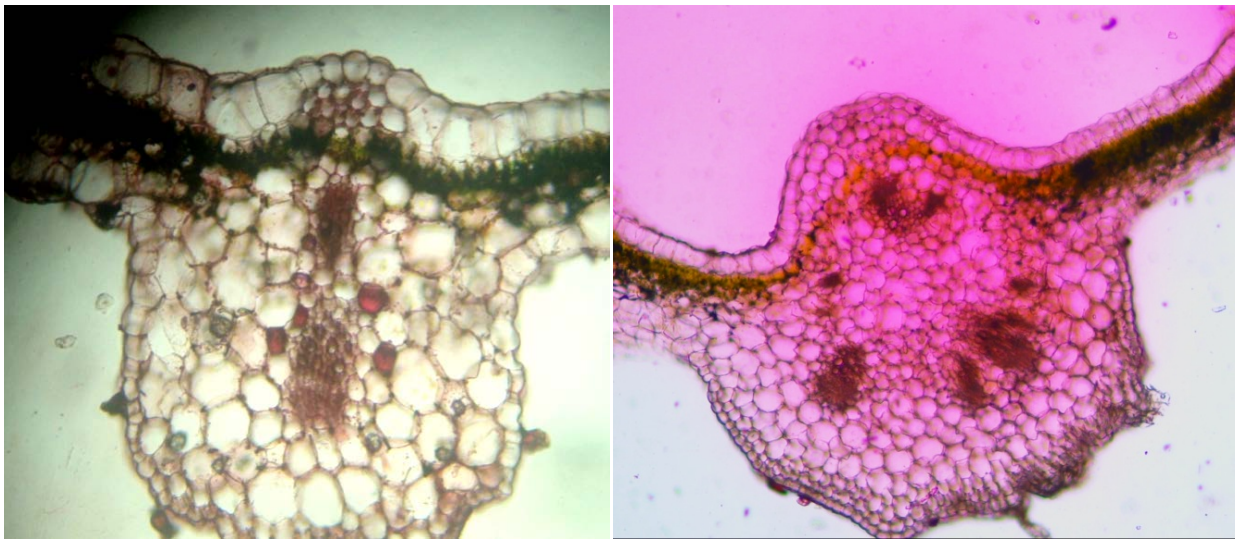
d e

Figure 3. Developmental stages of somatic embryogenesis of *Begonia malabarica*: (a) globular (b) heart shaped (c) torpedo (d) Shoot proliferation from embryogenic callus (e) Fully developed plantlets from somatic embryos

Figure 4: Hardening of tissue culture raised *Begonia malabarica* plants

a b

Figure 5. Cell suspension culture of *Begonia malabarica* from friable callus in MS medium fortified with 0.1mg L^{-1} 2,4-D



a b

Figure 6. (a) Leaf anatomy of *in vitro* plant ; (b) Leaf anatomy of *in vivo* plant

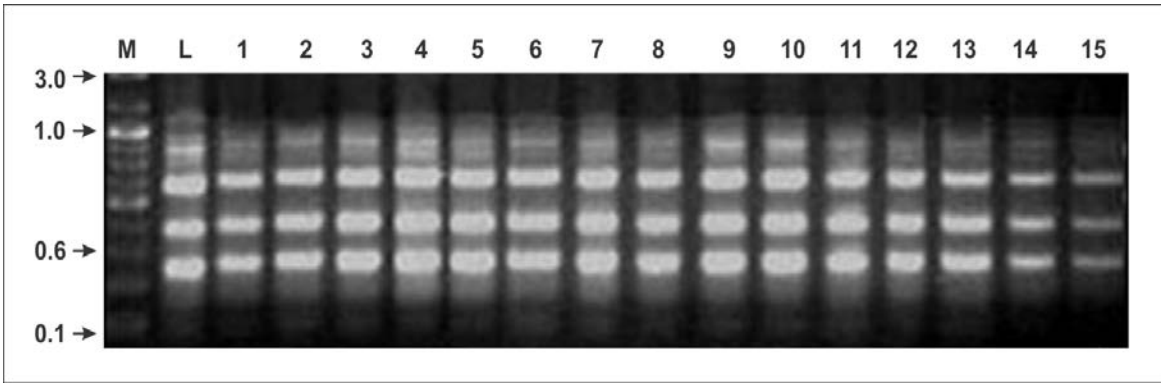


Figure 7. RAPD primer OPD-08 of *Begonia malabarica* showing monomorphic bands. (Markers in Kb unit)

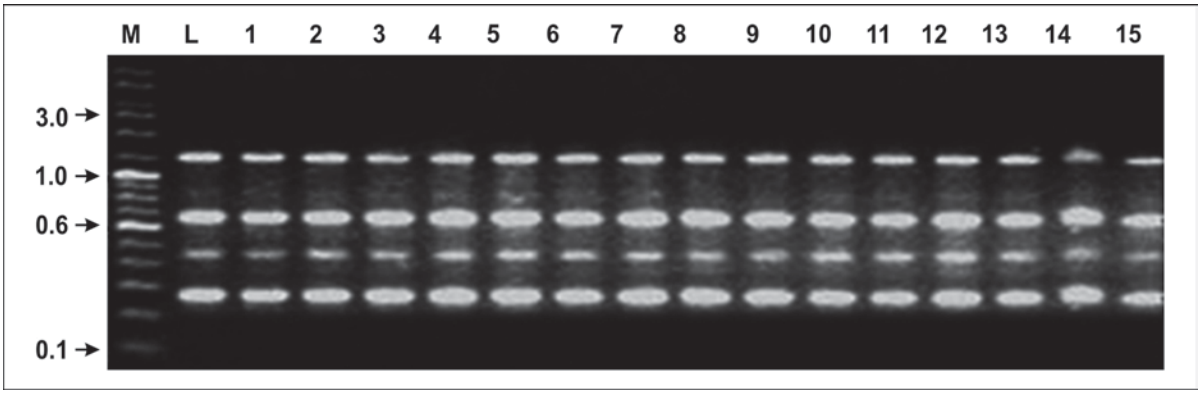


Figure 8. ISSR primer IG-18 of *Begonia malabarica* displaying monomorphic bands

(Markers in Kb unit)