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An efficient and rapid method of *in-vitro* propagation of *Bougainvillea* through nodal segments and clonal fidelity analysis using ISSRs

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Abstract
Bougainvillea is a thorny ornamental shrub with high aesthetic value belongs to Nyctaginaceae family. The usual propagation route through hardwood cuttings is strenuous. Here we report an efficient and rapid micropropagation protocol for *Bougainvillea* using nodal segments as explants. The MS media added with growth regulators at different concentrations and tested for their efficiency in culture establishment, multiple shoot development and root induction. The full-strength MS medium along with 1 mg/L of BAP was found to be ideal for culture establishment. Optimum culture multiplication was obtained in MS medium added with 1 mg/L of BAP and 1 mg/L of NAA. The shoots regenerated were efficiently rooted on full-strength MS medium with 2.5 mg/L of each IBA and NAA. The plantlets after ex-vitro hardening were transferred to pots and then to the soil, after five rounds of sub-culturing. The clonal fidelity of the micro-propagated plantlets was ensured with the ISSR primers UBC 815 and UBC 836 after each round of sub-culturing. The cost of micro-propagation was estimated and the cost per plantlet was found to be Rs. 20/-. Hence this rapid method is recommended for micro-propagation of *Bougainvillea* at a commercial large scale production.

Keywords: *Bougainvillea*, micro-propagation, MS media, clonal fidelity, commercial scale

Introduction

Bougainvillea (*Bougainvillea* spp.) is one of the most attractive ornamental plants in floriculture and this versatile plant belongs to Nyctaginaceae. It is commonly called as a 'paper flower' and the genus comprises about 18 species according to "The Plant List" (2017). Among these, *B. glabra*, *B. buttiana*, *B. spectabilis,* and *B. peruviana* are horticulturally important, typically seen as hedge, climber and bonsai (Kobayashi *et al.,* 2007) [9]. *Bougainvillea* is reported as a traditional medicinal plant with anti-inflammatory, anti-microbial, anti-cancer, anti-oxidant and anti-ulcer properties (Dhadde *et al*., 2021) [4].
Bougainvillea is usually propagated using hardwood cuttings, whereas the process is laborious and time-consuming (Ahmad *et al.,* 2007) [1]. The propagation of *Bougainvillea* is difficult and it will not produce seeds in tropical climate (Duhoky and Al-Mizory, 2014) [5]. Certain varieties of roof and leaf cuttings require mist conditions, along with root growth-promoting hormones for propagation (Hartman & Kester, 1989) [6]. Moreover, the success rate is very low, when the plants are propagated through cuttings. To meet the market demand for bougainvillae planting materials we need mass multiplication techniques and micropropagation techniques will be highly efficient. Kumari *et al*. (2016) [8] reported mass multiplication of Mahatma Gandhi and Refulgens through *in vitro* multiplication by using nodal sections with axillary buds as explants.
Jain *et al*. (2016) [7] attempted *in vitro* multiplication of Mahara cultivar using axillary bud with mediocre success. Chaturvedi *et al.* (1978) [3] successfully reported the shoot tip culture *Bougainvillea* through *in-vitro* propagation. Another study was carried out by Shah *et al.* (2006) [15] to standardize the mass multiplication of *Bougainvillea spectabilis* for commercial cultivation using shoot tips as explant. Duhoky and Al-Mizory (2014) [5] studied the effect of two culture media *viz*., MS medium and WPM medium in *Bougainvillea* to develop a protocol for rapid callus development using nodal explants and subsequent shoot regeneration.
A commercially viable micro-propagation protocol in *Bougainvillea* is needed to meet the demand for planting material. Occurrence of soma-clonal variations is the major problem associated with the plant cells when cultured in an artificial environment in the sub-clones.

Clonal fidelity analysis is important for commercial micro-propagation to check the genetic variations, if any, in the *in vitro* raised plantlets and mother plants. The ISSR (Inter Simple Sequence Repeat) marker serves as an important molecular marker for the assessment of genetic variability among plants (Alizadeh *et al.,* 2015) [2]. The present study was devised to establish a commercially viable micro-propagation protocol for *Bougainvillea* using nodal segments as explants and to ensure the plantlet's genetic stability using ISSR markers.

Materials and Methods

Culture establishment

Nodal segments of 1 to 2 cm from tender shoots were used as explants for *Bougainvillea* culture establishment. The collected explants were washed in running tap water for 30 min, followed by washing in% Bavistin (fungicide) with one drop of Tween-20 for 10 min. Further surface sterilization was carried out inside a laminar airflow cabinet. Then the explants were rinsed with sterile distilled water for 3 times to wash away the fungicide traces. These pretreatment explants were then surface sterilised with 0.1% mercuric chloride for 5 minutes after being treated with 70% ethanol for 30 seconds. The traces of sterilants were washed away using sterile water. Surface sterilized explants were cultured on MS media with 100 mg/L myo-inositol and 3% sucrose as carbon sources, as well as varied concentrations of growth regulators such as BAP and NAA (Table_1). Single nodal explants were inoculated vertically on media and cultures were incubated for 2 weeks at 25±2 ºC under 16/8 h light/dark (2400 lux.).

Culture multiplication

The established shoots were sub-cultured in MS medium supplemented with various combinations of BAP, NAA and kinetin for 3 weeks (Table_2).

Rooting and hardening

To induce roots, 4 to 5 cm long regenerated shoots were carefully separated from shoot clumps and individually placed to MS media with varied combinations of IBA (1-3 mg/L) and NAA (1-3 mg/L) (Table_3) for 3 weeks. After removing the *in vitro* rooted plants from the culture bottles, the roots were rinsed with distilled water to remove the agar before being put into pots containing a 3:1:1 combination of coco peat, Soilrite, and vermiculite. For two weeks, the potted plants were placed in an amidst chamber for primary hardening. The healthy plants were transferred to pots with potting mixture and maintained in the net house for one month for secondary hardening.

Clonal fidelity analysis

Fresh young tender leaves of tissue cultures raised *Bougainvillea* plants from the second subculture to the 5th subculture and the leaves of the mother plant were sampled to assess the genetic stability. Genomic DNA was isolated as per the CTAB method by Rogers and Benedich (1994) [13] with some modifications. The DNA quality was assessed on 1% agarose gel and the DNA was quantified using a Nanodrop spectrophotometer. The extracted DNA was diluted to a final concentration of 25ng/µl.

The ISSR assay was performed to ensure clonal fidelity in the micro-propagated plantlets. The ISSR primers UBC 815 and UBC 836 were used for amplification. The 20µl of reaction mixture consisted of 2µl of template DNA, 2µl of primer, 1X assay buffer, 1.8 µl of $MgCl_2$, 1.8µl of dNTP mix and 0.4µl of *Taq* DNA polymerase enzyme. The template DNA was amplified in the PCR with an initial denaturation at 94°C for 5 min, followed by 35 cycles denaturation at 94°C for 30 sec, primer annealing at 52°C for 1 min and extension at 72°C for 2 min, followed by final extension at 72°C for 7 min. Then PCR products were separated on a 2% agarose gel by electrophoresis using 1X TAE buffer. The obtained amplification pattern of UBC 815 and UBC 836 was observed for the analysis of clonal fidelity in micro-propagated *Bougainvillea*.

Results and Discussion

Culture establishment

We observed 33.33−81.33% culture establishment with the nodal explants of a *Bougainvillea* in different media combinations. Culture initiation was observed within 2−3 weeks of inoculation. The full MS media supplemented with 1 mg/L of BAP showed the best response. Here 81.33% efficiency was observed that reduced to 70% at double the dosage of BAP. The lowest number of shoots (33.33%) was obtained on MS medium with 1 mg/L concentration of NAA, devoid of BAP (Table_4).

Culture multiplication

For shoot multiplication and proliferation of the cultures, the established shoots from MS media with 1 mg/L of BAP were transferred into MS media with ten different combinations of NAA, BAP and kinetin. In the first round of sub-culturing, we observed optimum shoot proliferation in seven out of the ten media tested. The three media combinations with kinetin (MM5, MM9, MM10) failed to induce multiple shoots (Table_4).Our observation that media without kinetin were showing good shoot multiplication was on par with the results of similar studies. Ramanujan *et al.* (1999) [11] reported that BAP was more efficient than kinetin in inducing multiple shoots from nodal explants of Amaranthus. Nagarajan *et al.* (2006) [14] also studied the direct shoot regeneration from nodal explants of *Bougainvillea* and observed that MS medium with 2 mg/L of BAP was ideal. They also reported that kinetin was less efficient in inducing shoot multiplication. Observations from our third subculture showed that MM3 (MS medium with 1 mg/L of BAP and 1 mg/L of NAA) showed the highest multiple shoot proliferation. Full-strength MS medium was found to be the ideal medium for shoot multiplication than half-strength medium with growth regulators such as BAP and NAA. Among the seven media that showed optimal shoot proliferation during the first round of subculture, MM8 (half-strength MS with 2 mg/L BAP and 0.5 mg/L NAA) had the least efficacy. In many plants, BAP is reported to be an ideal hormone for shoot multiplication (Nagarajan *et al.,* 2006; Jain *et al*., 2016) [14, 7].

Rooting and hardening

For induction of roots, 4-5 cm long shoots with leaves were removed from shoot clumps developed from MM3 medium and individual shoots were transferred to various rooting media (full-strength MS supplemented with IBA and NAA). Among all these treatments, the maximum number of roots were observed in MR4 media (full strength MS medium with 2.5 mg/L each IBA and NAA) within 3 weeks (Table_4). In addition, we observed better quality of roots in terms of root thickness and root length in this media. Further increase in the concentration of IBA and NAA (3 mg/L each) in the culture

medium showed lesser root growth and quality attributes. The combination of NAA and IBA for root induction was reported by Singh and Syamyl (2001) [16] in the case of axillary shoot proliferation in rose. Also, this combination of two auxins proved superior for root induction in *Bougainvillea* (Singh *et al.,* 2013; Jain *et al.,* 2016) [7, 17]. Shah *et al.,* 2006 [15] reported 100% root induction in *Bougainvillea* in half-strength MS medium supplemented with two auxins (IBA and NAA 2.5 mg/L each).

The healthy rooted plantlets were subjected to primary hardening for 2 weeks and the hardened plants were transferred to potting mixture for 4 weeks for secondary hardening. Plants grew well and flowered within 1-2 months after acclimatization on the soil. The different stages in the *in vitro* propagation of *Bougainvillea* are represented in Figure_1.

Clonal fidelity analysis

Screening of the mother plant and tissue culture plantlets of *Bougainvillea* revealed that banding profiles obtained with UBC 815 and UBC 836 primers were identical for the mother plant and tissue culture raised plantlets (Figure_2). The primer UBC 815 produced a uniform banding pattern with 3 bands in four stages of tissue-cultured plants (subcultures 2, 34 and 5) and the mother plant. Similarly, primer UBC 836 also produced a uniform banding pattern with 3 bands in four stages of tissue-cultured plants (subcultures 2, 3, 4, 5) and the mother plant. No polymorphic bands were detected. This ensures the genetic stability of the plantlets. The ISSR markers have been used to identify and assess the level of clonal fidelity and genetic analysis of tissue culture-raised plants (Rastogi *et al.,* 2019) [12].

Micro-propagation protocols in *Bougainvillea* have been reported in many studies. In our study, we got optimum culture establishment in 1mg/L BAP, culture multiplication in 1mg/L BAP and 1mg/L NAA and rooting in 2.5mg/L IBA and 2.5mg/L NAA combinations. Jain *et al.* (2016) [7] tried both shoot tip and nodal sections for micro-propagation in *Bougainvillea* and reported the hormone combination of 5mg/L BAP and 1mg/L NAA for shoot proliferation and 1.5mg/L GA3 for shoot elongation. Shah *et al.* (2006) [15] also reported regeneration in *Bougainvillea* through shoot tip explants and observed the ideal combination of 0.25 mg/L BAP and 0.1 mg/L NAA. In our study, 1 mg/L BAP was found to be ideal for culture establishment while 1 mg/L BAP along with 1 mg/L NAA was optimum for culture multiplication. We also performed the clonal fidelity analysis to ensure the genetic stability of micro propagated plantlets and have also worked out the commercial viability of our protocol.

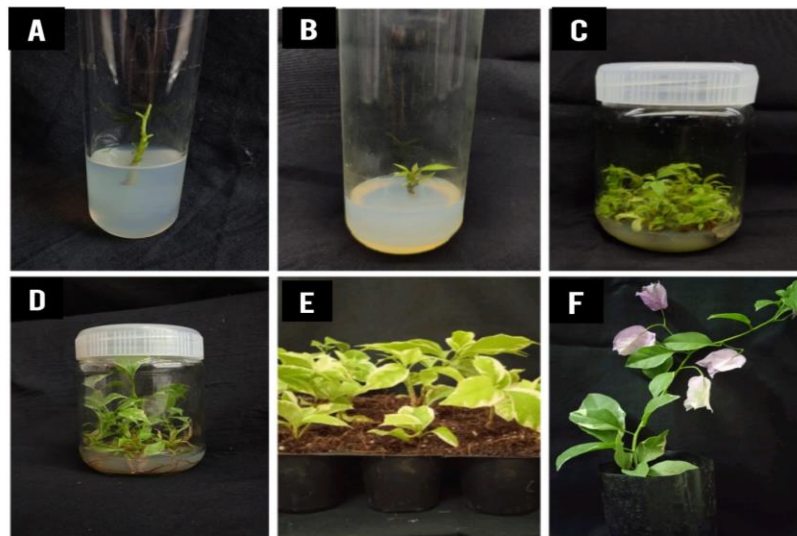

Fig 1: Stages of *in vitro* regeneration and acclimatization of *Bougainvillea*

A) Inoculation of nodal explant in MS media supplemented with 1 mg/l BAP, B) Culture establishment, C) Multiple shoot proliferation in full strength MS media supplemented with 1mg/l BAP and 1mg/l NAA, D) Root induction in full strength MS media supplemented with 2.5 mg/l IBA and 2.5 mg/l NAA, E) Primary hardening in protrays, F) Plants after acclimatization.

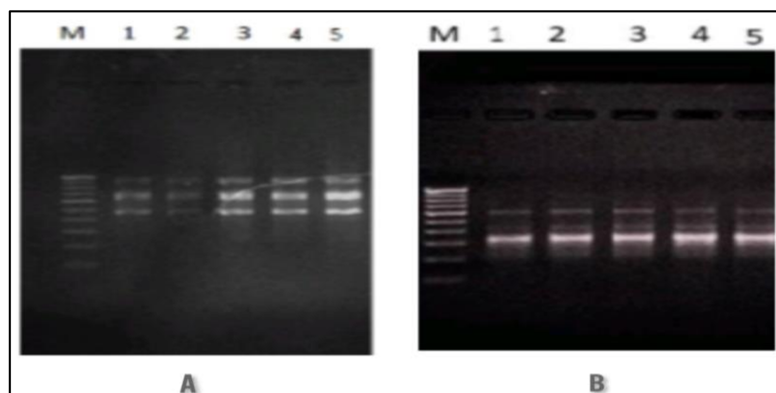

Fig 2: Clonal fidelity analysis in micro propagated *Bougainvillea* using ISSR markers

Banding pattern of mother plant (1) and four stages (subcultures 2, 3, 4, 5) of tissue cultured *Bougainvillea* plants (represents 2, 3, 4, 5) generated by ISSR marker (A) UBC 815 primer, and (B) UBC 836 primer. M represents 100bp DNA ladder.

Table 1: Media combinations used for culture establishment in *Bougainvillea*

Media code	Basal media (MS)	Growth regulators (mg/L)		Sucrose
		BAP	NAA	
ME1	Full	0.5	—	3
ME2	Full	1	—	3
ME3	Full	2	—	3
ME4	Full	1.5	1	3
ME5	Full	—	1	3

Full-strength MS media with different combinations of BAP and NAA were used
ME- Establishment media

Table 2: Media combinations used for shoot proliferation and multiplication in *Bougainvillea*

Media code	Basal media (MS)	Growth regulators (mg/L)			Sucrose
		BAP	NAA	Kinetin	
MM1	Full	0.5	1	—	3
MM2	Full	1	0.5	—	3
MM3	Full	1	1	—	3
MM4	Full	2	1	—	3
MM5	Full	—	—	1	3
MM6	Half	1	1	—	3
MM7	Half	1.5	1.5	—	3
MM8	Half	2	0.5	—	3
MM9	Half	—	—	0.5	3
MM10	Half	—	—	2	3

Half and Full-strength MS media with different combinations of BAP, NAA and Kinetin were used
MM- Multiplication media

Table 3: Media combinations used for root induction in *Bougainvillea*

Media code	Basal media (MS)	Growth regulators (mg/L)		Sucrose
		IBA	NAA	
MR1	Full	—	—	3.0
MR2	Full	1	1	3.0
MR3	Full	2	1.5	3.0
MR4	Full	2.5	2.5	3.0
MR5	Full	3	3	3.0

Full-strength MS media with different combinations of IBA, NAA and Kinetin were used
MR- Rooting media

Table 4: *In vitro* response of *Bougainvillea* culture in different media combinations

Type of media	No. of explants per triplicate	Culture establishment	Type of media	No. of sprouts subcultured per triplicate	Shoots induced at first subculture	Shoots induced at third subculture	Type of media	Culture for root induction per triplicate	No. of rooted shoots
ME1	25	14.3±0.9	MM1	40	39.3±0.3	80±0	MR1	25	19±1
ME2	25	20.3±0.7	MM2	40	35.7±2.9	71±14.1	MR2	25	16.7±0.3
ME3	25	18±1	MM3	40	39.7±0.3	134.3±5.4	MR3	25	21±1.7
ME4	25	15.7±0.7	MM4	40	38.7±0.3	60.3±3.7	MR4	25	25±0
ME5	25	8.3±0.9	MM5	40	0±0	0±0	MR5	25	16.3±1.3
			MM6	40	39.3±0.3	72.7±7.6			
			MM7	40	39.7±0.3	56.7±12			
			MM8	40	39±0.6	50.3±5.8			
			MM9	40	0±0	0±0			
			MM10	40	0±0	0±0			

The response during various stages like culture establishment, culture multiplication and rooting are indicated. The experiment was conducted in triplicate and the mean is presented here as mean±SE.
ME- Establishment media
MM- Multiplication media
MR- Rooting media

Conclusion

The protocol reported here for mass multiplication of *Bougainvillea* using nodal segments is very efficient. The percentage of plants lost due to contamination is less than 10 percent during the culture establishment stage showing the efficiency of our surface sterilization method. The cost of production of plantlets was estimated and was found to be Rs. 20 /- per plantlet. We could sell the plantlets after secondary hardening at a cost of Rs. 100/- plantlet from our commercial tissue culture unit, thus showing the economic viability of our

protocol. With our protocol, we could obtain around 650 plantlets in 3.5 months from a single nodal explant; hence, the method can be recommended for the commercial scale multiplication in *Bougainvillea*.

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Conflict of Interest
The authors declare that they have no conflict of interest.

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