In Vitro Multiplication from Nodal Explant of Roseapple and Genetic Purity Study of Conserved Plantlets Using Molecular Marker

Argha Chakraborty* and Prakash Kanti Das

Department of Agricultural Biotechnology, Ramakrishna Mission Vivekananda Educational and Research Institute, RKM Ashrama, Narendrapur, Kolkata, India

*Corresponding author: argha100chakra@gmail.com

Received: 06-01-2019 Revised: 03-04-2019 Accepted: 24-05-2019

ABSTRACT

In vitro multiplication, an exceptional replacement for conventional propagation practices as it produces maximum plantlets in minimum space and time. In vitro multiplication in rose apple was attempted through direct organogenesis of nodal explant. During shoot multiplication the highest numbers of shoots with the maximum number of leaves were achieved in full MS as well as in half MS with 4 mg/l BAP supplementation but half MS were recommended for better survival of plants as it lowered the polyphenol exudates interfering plant survival. In vitro root induction was achieved within two weeks of shootlet cultured on half MS with IBA at 6 mg/l concentration. Shoot multiplication from nodal explant produced on an average 30 plantlets from a single seed within 4-5 months. In vitro produced as well as conserved plantlets showed no genetic differences when it is compared to in vivo regenerated plants by using RAPD markers.

Keywords: Roseapple, nodal explant, MS, PGR, RAPD

MATERIALS AND METHODS

Ripened fruits were collected from different growing areas of South 24 Parganas, West Bengal, India. They were thoroughly washed, cleaned, dried and imbibed in water for overnight and surface sterilized with 1% (v/v) savlon + 1% (v/v) Tween 20 for 30 minutes, washed 4 times in distilled water, also treated with 1% (w/v) Bavistin for 10 minutes, washed for 4 times in distilled water. They were then treated with 0.1% (w/v) Mercuric Chloride
HgCl$_2$) for 10 minutes and washed for 4 times with distilled water.

Surface sterilized seeds were cultured in 500 ml culture vessels containing 40 ml of MS semisolid media with 30g/l sucrose, 100 mg/l myoinositol without any PGR.

Nodal explants of 1.5-2 cm length were excised from in vitro regenerated 70 days old plants. They were inoculated in the 500 ml culture vessels containing 40 ml of full strength MS with 30g/l sucrose, 100 mg/l myoinositol and different concentrations of BAP (0, 1, 2, 4, 6 and 8 mg/l) and in half strength MS with different concentrations of BAP as in MS full strength. Both MS and half MS were supplemented with 30 mg/l PVP as suggested by Quraishi et al. to control polyphenol exudates. Regenerated shootlets were transferred to the half strength MS media with 6 and 8 mg/l IBA for root induction.

During the course of study the culture vessels were kept at 25 °C under standard cool fluorescent tubes providing 60 µmol/m$^2$/s irradiance at a 16 hour photoperiod.

To test the genetic fidelity, one year old plantlets maintained in MS through subcultures (5-6 weeks interval) were compared with (i) in vivo raised plants, (ii) in vitro regenerated plants afresh, (iii) regenerants in vitro maintained for 70 days in MS with two subcultures of 5 weeks interval. Genomic DNA was isolated according to the procedure described by Allen et al. The concentration of DNA was determined by a UV-vis spectrophotometer (Thermo scientific, GENSYS 10S) and quality of the DNA was assured through electrophoresis on 0.8% agarose gel. For PCR amplification, 10µl optimized PCR mixture was prepared comprising of 20 ng template DNA, 40ng primer, 1x PCR buffer, 250 µM dNTPs, 1.5 mM MgCl$_2$, 0.1 U Taq polymerase. The PCR amplification profile was set as- 94 °C for 2 min (initial denaturation), (94 °C for 1 min, 35 °C for 1 min, 72 °C for 2 min) 35 cycles, final extension at 72 °C for 10 min. A total of 10 RAPD primers were randomly employed from the OPA series and OPB series. The details of the primers are given in the Table 1. Aliquots of 5 µl PCR products, along with DNA ladder were resolved by 1.5% agarose gel in 1x TAE buffer, stained with Ethidium Bromide (EtBr) (0.5 µg/ml). Gels were scanned and analyzed using life science software. The sizes of the PCR product were compared to the molecular size standard 100 bp DNA ladder (Chromous Biotech, India).

The collected data were analysed using one way analysis of variance (ANOVA). ANOVA of % survival was done after square root transformation of % data. Treatment means were compared based on Least Significant Difference (LSD) through AGRES (version 7.0) software package.

**RESULTS AND DISCUSSION**

**Plantlet regeneration and multiplication**

Bud break (Fig. 1A) occurred after 2-3 weeks of culture. It was almost 4-5 days earlier in half MS than full MS. All shoot growth characters were recorded after 7-8 weeks of bud break. A gradual increase was seen in growth characters up to 4 mg/l concentration of BAP both for full MS and half MS. Thereafter with increase of BAP concentrations (6 and 8 mg/l) a decline on growth characters was observed. Significantly at 4 mg/l BAP both full strength and half strength recorded maximum shoot length, leaf number and other shoot characters. However numbers of leaves were significantly higher in full MS. Under 4 mg/l BAP supplementation, majority of the shoot characters in full MS and half MS ran in parallel (Table 2, Fig. 1 B, C).

Shootlet survival under different concentrations of BAP was compared between full MS and half MS. ANOVA indicated that MS strength, BAP concentrations and their interactions were significant. Shootlet survival percent in half MS with 4 mg/l BAP was significantly higher than full strength MS with same BAP supplementation which was around 86% in half MS but only 20% in full MS (Table 3).

Root induction was recorded within 2-3 weeks of culturing on half MS supplemented with either 6 or 8 mg/l IBA. It was found that both 6 mg/l and 8 mg/l IBA produced 12-15 robust roots of about 1 cm length within 7-8 weeks of culture (Fig. 1D). No significant differences between these two concentrations were found. It can be suggested that 6 mg/l IBA is good enough to induce root in vitro. This confirms earlier observation of Prashanta et al.
markers which do not require any knowledge of DNA sequence of targeted genome. The primer will bind randomly in the sequence. However it relies on a large, intact DNA samples and its resolving power is also much lower than target specific DNA comparison methods. Because of its simplicity and random DNA binding capacity Shakya et al. found it useful to characterize different genotypes of *S. cumini*, another popular species of *Syzygium* genus in which roseapple belongs. Rani et al. used RAPD markers to test genetic fidelity of meristem derived plants raised in vitro. In the present study out of 10 RAPD deca primers, 4 primers show consistent banding pattern and those 4 primers considered for study. Altogether 29 consistent monomorphic bands were recorded per sample. Eventually 116 bands from different samples were derived under assessment and the comparison displayed no discrepancy (Fig. 1E). Thus, it can be concluded that derived planlets through direct organogenesis in vitro and conserved plantlets in vitro, atleast for a period of 1 year do not produce any genetic changes. However with the inherent limitation of RAPD as molecular marker present observations need to be considered.

**Table 1:** List of primers with their sequences and melting temperatures (Tm)

| Sl. No. | Name of the primer | Sequences         | Tm(°C) |
|--------|--------------------|-------------------|--------|
| 1      | OPA-2              | TGCCGAGCTG        | 40.7   |
| 2      | OPA-4              | AATCGGGCTG        | 35.1   |
| 3      | OPA-7              | GAAACGGGTG        | 33.2   |
| 4      | OPA-8              | GTGACGTAGG        | 31.1   |
| 5      | OPA-10             | GTGATCGCAG        | 33.1   |
| 6      | OPA-17             | GACCGCTTGT        | 35.7   |
| 7      | OPA-20             | GTTGCAGATCC       | 33.5   |
| 8      | OPB-1              | GTTCGCTCC         | 35.8   |
| 9      | OPB-2              | TGATCCCCTGG       | 32.2   |
| 10     | OPB-4              | GGACTGGAGT        | 32.2   |

**Fig. 1:** Successful *in vitro* shoot multiplication of *S. jambos* and genetic fidelity study. (A) Bud break after 2-3 weeks. (B,C) Shootlets in full MS and half MS respectively showing no significant difference. (D) Shootlet with robust root growth after 7-8 weeks in half MS+6mg/l IBA. (E) Amplified RAPD fragments of different samples using primer OPA-10; [L- 100bp+ ladder, 1- *in vivo* sample, 2- *in vitro* sample, 3- *in vitro* sample (after 2 subculture), 4- *in vitro* sample (after one year maintenance)]
We have seen that most shoot growth characters ran parallel in both half and full strength MS. But in case of shootlet survival, half MS showed far better result than full MS. Such observation is not unexpected. The reduced MS strength to half inhibits polyphenol exudates which helps promote survivality. This is also observed by Qureshi et al. Apart from this, bud break also took place earlier in half MS, which shortens the time period for multiplication. BAP concentration is proved to be another key player in plantlet regeneration and multiplication. BAP concentration at 4 mg/l showed the best result in terms of shootlet growth characters, while comparing with others. Thus, considering both shootlet growth and survival, half MS with 4 mg/l BAP appeared to be desirable media formulation. Interestingly Prashanta et al. also observed 4 mg/l BAP as favourable concentration for shootlet growth of S. jambos, but his study was confined to full MS.

Different events over different time schedule during the course of nodal explant multiplication in vitro (bud break-shootlet-rooting) have been summerized (Table 4). It becomes evident that it took around 16-20 weeks i.e. around four and half months to raise complete plantlets from the nodal explants. It is to be noted that as many as 7-8 shootlets were induced in vitro in MS without PGR from a single seed. During the course of the study, altogether 4 nodal explants were derived from a single shoot. Based on these facts, it is possible to propagate as many as 28-32 plants from a single seed through nodal explants multiplication in vitro. This in vitro multiplied shootlets did not show any genetic variability as confirmed by the genetic fidelity test. So, from these observations, it can be concluded, roseapple can be multiplied within a short time span and conserved in vitro (atleast one year) for continuous supply to the farmers without hampering its genetic makeup.

**ACKNOWLEDGEMENTS**

The authors duly acknowledge the facilities and help provided by IRDM faculty centre, RKMVERI, Narendrapur, Kolkata, West Bengal. We also thank assistances received from different quarters during the course of the study.
REFERENCES

1. Schmitt, L. and Riviere, J.N. 2002. Comparative life-history traits of two *Syzygium* species (Myrtaceae): one invasive alien in La Réunion, the other native. *Acta Botanica Gallica*, 149(4): 457-466.

2. Litz, R.E. 1984. *In vitro* responses of adventitious embryos of two polyembryonic *Eugenia* species [*Eugenia jambos, Eugenia malaccensis*, propagation, somatic embryogenesis, tissue culture] *HortScience*.

3. Prashanta, K.G., Sathyanarayana, B.N., Mathew, D. and Sondur, S.N. 200. *In vitro* Callus Induction and Plantlet Regeneration in Roseapple (*Syzygium jambos* L.), *Journal of Plant Biology-New Delhi*, 30(1): 99-102.

4. Quraishi, A. and Mishra, S.K. 1998. Micropropagation of nodal explants from adult trees of *Cleistanthus collinus*, *Plant Cell Reports*, 17(5): 430-433.

5. Allen, G.C., Flores-Vergara, M.A., Krasynanski, S., Kumar, S. and Thompson, W.F. 2006. A modified protocol for rapid DNA isolation from plant tissues using cetyltrimethylammonium bromide, *Nature Protocols*, 1(5): 2320-2325.

6. Shakya, R., Siddiqui, S.A., Srivatawa, N. and Bajpai, A. 2010. Molecular Characterization of Jamun (*Syzygium cumini L. Skeels*) Genetic Resources, *International Journal of Fruit Science*, 10(1): 29-39.

7. Rani, V. and Raina, S.N. 2000. Genetic fidelity of organized meristem-derived micropropagated plants: a critical reappraisal. *In Vitro Cellular & Developmental Biology-Plant*, 36(5): 319-330.
