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Clonal Fidelity of *In Vitro* Propagated Orchid Cultivars *Dendrobium* 'Sonia' and *Dendrobium nobile* var. 'Emma White'

Kamni Kumari[1], Kundan Kishor Rajak[1], Chandan Kishore[2*] and Mithilesh Kumar[1]

[1]Department of Agricultural Biotecnology and Molecular Biology,
Rajendra Agricultural University, Pusa, Samastipur- 848125, India

[2]Department of Plant Breeding and Genetics, Bihar Agricultural University, Sabour, Bhagalpur-813210, India

*Corresponding Author E-mail: chandanpbg@gmil.com

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ABSTRACT

Micropropagated plantlets of two orchid cultivars namely Dendrobium 'Sonia' and Dendrobium nobile var. 'Emma White' were analyzed for genetic fidelity by RAPD markers. The in vitro raised plantlets were maintained repeatedly by in- vitro subcultures. Out of 20 primers utilized during present investigation, amplification was achieved with all primers. But reproducible amplification protocol was standardized only for 8 primers namely, OPL-02, OPL-03, OPL-8, OPL- 11, OPN-01, OPN-06, OPN-09, OPN-10. The amplified products were monomorphic across all the selected micropropagated plants and were similar to the mother plant. So, the RAPD analysis of micro propagated plant along with their original cultivar with eight decamer primers did not showed any variation. Hence, it confirmed the genetic uniformity of micropropagated plantlets in the present study.

Key words: *Orchid, Tissue Culture, MS media, RAPD*

INTRODUCTION

The orchid is one of the most fascinating flowers; known for its eye pleasant colors, shape and size. It holds a significant position in cut flower industry due to its attractiveness, long shelf life, high productivity, right season of bloom, easy in packing and transportation. Propagation of orchids through conventional means has lost its significance in commercial venture, because vegetative multiplication through division and back bulb culture is rather slow and yields only a meager number of plantlets even after 5-6 years (Pathak *et al.*, 2011), thus hampering large-scale commercial propagation. Micropropagation has the ability to massively produce clone plants at low costs and has been successfully employed for the commercialization at an industrial level. However, commercialization of its *in vitro* propagated plantlets requires genetic stability for true to the type to assure its quality and appearance. The clonal copies may change due to stresses imposed on the plants under *in vitro* conditions and the regenerated plantlets loose their clonal identity probably due to somaclonal variations.

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The confirmation of true-to- the type can be achieved by test of genetic fidelity to discard the undesired variant plants.

Molecular marker technique can be used to test the genetic fidelity of *in vitro* propagated plants. The polymerase chain reaction (PCR) based techniques such as RAPD (Random Amplified Polymorphic DNA) and AFLP (Amplified Fragent-Length Polymorphisms) have been found immensely useful in establishing the genetic fidelity of *in vitro* regenerated plants. The RAPD technique has many advantages such as simplicity and rapidity of analysis, low cost, availability of a large number of primers and the requirement of small amount of DNA for analysis (Williams *et al.*, 1990; Huff *et al*., 1993; Ge *et al.*, 1999; Nybom and Bartish, 2000; Kingston *et al.*, 2004). RAPD analysis using PCR in association with short primers of arbitrary sequence has been demonstrated to be sensitive in detecting variation among individuals (Sheidai *et al.*, 2010). RAPD technique is more suited to orchids since very little is known about the genetic diversity within the natural populations (Xiaohong *et al*., 2007).Thus, the present study was formulated with the objectives to test the genetic fidelity of micropropagated plants of orchid cultivars namely *Dendrobium* 'Sonia' and *Dendrobium nobile* var. 'Emma White' by RAPD markers.

MATERIALS AND METHOD

In the present experiment micropropagated plants of orchid cultivars namely *Dendrobium* 'Sonia' and *Dendrobium nobile* var. 'Emma White' were raised through explants *viz*. shoot apex, nodal stem, young leaf, immature embryo and root tip. These plantlets were then subjected for test of genetic fidelity by RAPD markers. The genomic DNA was extracted from orchid leaf samples collected from field grown mother plant and their tissue cultured plants of two different genotypes by using CTAB method proposed by Dellaporta *et al.*, 1983 with slight modifications and the use of liquid nitrogen was omitted followed by RNase treatment. Random primers for PCR were synthesized by Operon technologies, USA. The details of amplified primers used in present study are given in Table 1. The PCR reaction mixture and standard PCR conditions of RAPD are given in Table 2 & 3. The RAPD decamer Primers were obtained from Eurofins mwg/operon. The primer vials were centrifuged before and after the addition of TE buffer to the vials. Mentioned volume of the TE was added to each vial so as to obtain the desired concentration of the primer stock solution (100pmol/µl). Diluted primers were stored at -20°C. *In vitro* DNA amplification was carried out using standard protocol of polymerase chain reaction (PCR) adjusted to laboratory conditions with known 20 primers. The PCR reaction was carried out in sterile 0.5 mm thin walled PCR tubes (Axygen Scientific Pvt. Ltd. Union City, USA) using 20 µl reaction mixtures. The amplification was carried out in Eppendorf thermocycler gradient 96 (lid temperature 105^0C). For different primers, annealing temperatures used were approximately 5°C below from their respective Tm (provided by company). Tm values were also verified by the formula

$$Tm = 4 (G + C) + 2 (A+T)$$

The RAPD amplified product with expected product size above 100 bp was subjected to 1.2% agarose gel electrophoresis at 100 V for 1 hr 15 min and then visualized and documented in gel documentation system (Bio-rad). The size of amplified fragments was estimated with 100 bp ladder (Fermantas).

RESULT AND DISCUSSION

In the present experiment extracted DNA was confirmed by electrophoretic analysis and a single sharp band of high molecular weight

was obtained, which were used for analysis of genetic variation through RAPD markers. Out of 20 primers utilized during present investigation, amplification was achieved with all primers. But reproducible amplification protocol was standardized only for 8 primers namely, OPL-02, OPL-03, OPL-8, OPL- 11, OPN-01, OPN-06, OPN-09, OPN-10. The primer pairs generating clear and reproducible patterns of DNA profile were selected for further study. The polymorphism showed by primers was recorded on the basis of presence or absence of band, number of bands and position of bands in relation to the position and size of the ladder (Table 4, Fig.1 & 2). No variations were observed between regenerated plants and field plants grown in field. The genetic similarity of two selected cultivars and their tissue cultured plant were estimated. The similarity was recorded on the basis of presence or absence of RAPD bands and the band score was used for computing similarity coefficient with respect to size of the amplified product generated from the targeted region of genome. The amplified products were monomorphic across all the selected micropropagated plants and were similar to the mother plant.

Earlier also RAPD and ISSR markers were successfully employed in bamboo species (Agnihotri et al., 2009; Singh et al., 2013), apple (Gupta et al., 2009), Jalamdasa (Chandrika and Rai, 2009), Acacia mangium (Nanda et al., 2004), Stevia rebaudiana (Lata et al., 2013) and many more.

Table: 1. Details of RAPD decamer primers.

Primer	Sequence (5´-3´)	Annealing Temperature Ta (º C)
OPL-02	TGGGCGTCAA	27
OPL-03	CCAGCAGCTT	27
OPL-08	AGCAGGTGGA	27
OPL-11	ACGATGAGCC	30
OPN-01	CTCACGTTGG	30
OPN-06	GAGACGCACA	27
OPN-09	TGCCGGCTTG	27
OPN-10	ACAACTGGGG	30

Table: 2. Composition of the PCR reaction mixture

Reagent	**Stock concentration**	**Working concentration**	**Vol. for 1 reaction**	**Vol. for 8 reaction**	**Supplier**
Primer	100pmol/µl	50 pmol/µl	0.8 µl	6.4µl	Eurofins mwg/operon
dNTPs	2mM	1.5mM	1.6µl	12.8µl	Fermentas
$MgCl_2$	25 mM	3.5 mM	0.8 µl	6.4µl	Fermentas
Taq Polymerase	5U/µl	-	0.96µl	7.68µl	Fermentas
Assay buffer	10x buffer	1x	2µl	16µl	Fermentas
Nuclease free Water	-	-	9.84µl	78.72µl	Fermentas
Template DNA	-	-	4 µl	32µl	-
Total			20 µl	160 µl	

Table: 3. Standard PCR conditions for RAPD reaction.

Sl. No.	**Reaction condition**	**Time and Temperature (°C)**
1.	Initial Denaturation	3 min. at 94°C
2.	30 cycles of	
	a)Denaturation	40 sec. at 94°C
	b)Annealing	40 sec. at variable (27-30°C)
	c)Extension	2 min. at 72°C
3.	Final Extension	10 min. at 72°C

Table 4. Details of number of bands and their size range obtained by RAPD primers

Sl. No.	**Primer**	**Total no. of bands**	**Range of Amplified Product (base pair)**
1.	OPT-02	5	100-1000
2.	OPT-03	4	300-1500
3.	OPT-08	6	500-3000
4.	OPT-11	5	300-1000
5.	OPN-01	6	400-1000
6.	OPN-06	6	300-1200
7.	OPN-09	8	250-800
8.	OPN-10	4	200-1000
	Total	44	

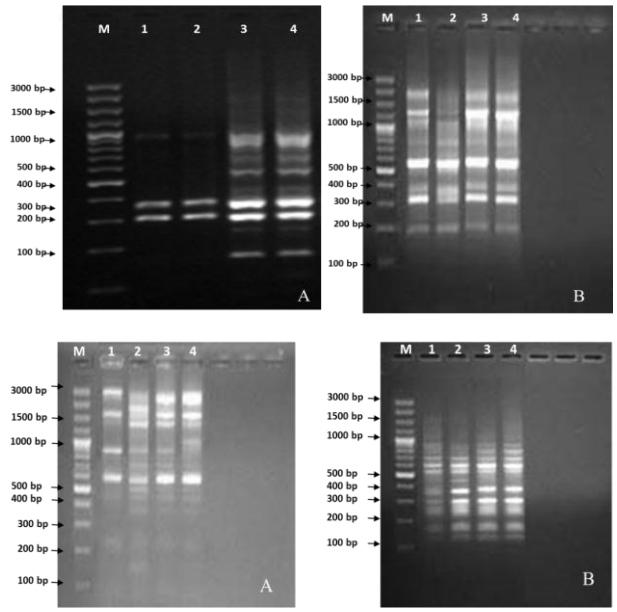

Fig.1 : RAPD-PCR amplification product of 2 different orchid verities having both Mother Plant (MP) and randomly selected their Tissue Culture Plant (TP) using primers OPL-02 (A), OPL-03 (B), OPL-08 (C) and OPL-11 (D). Lane M represents GeneRuler 100 bp Plus DNA Ladder, 1 – *Dendrobium* ;Sonia' (MP), 2 - *Dendrobium* ;Sonia' (TP), 3 – *Dendrobium nobile* var 'Emma White' (MP), 4 - *Dendrobium nobile* var 'Emma White' (TP).

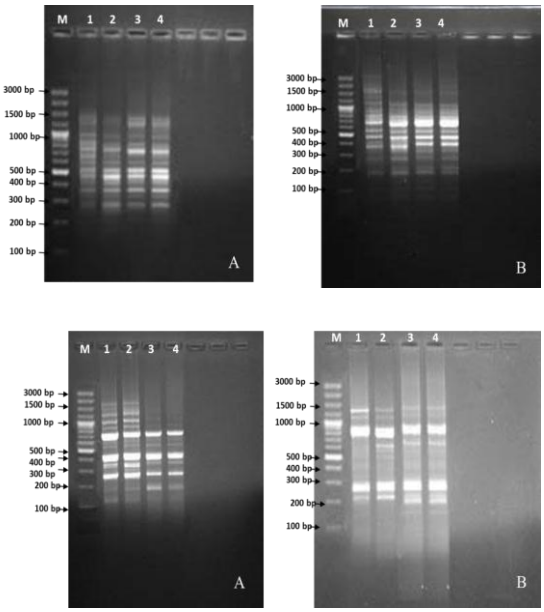

Fig.2: RAPD-PCR amplification product of 2 different orchid verities having both Mother Plant (MP) and randomly selected their Tissue Culture Plant (TP) using primers OPN-01 (A), OPN-06 (B), OPN-09 (C) and OPN-10 (D). Lane M represents GeneRuler 100 bp Plus DNA Ladder, 1 – *Dendrobium* ;Sonia' (MP), 2 - *Dendrobium* ;Sonia' (TP), 3 – *Dendrobium nobile* var 'Emma White' (MP), 4 - *Dendrobium nobile* var 'Emma White' (TP).

CONCLUSION

production of monomorphic bands by in vitro raised plantlets and mother plant of orchid cultivars namely *Dendrobium* 'Sonia' and *Dendrobium nobile* var. 'Emma White' confirmed the genetic uniformity of micropropagated plantlets in the present study. Since no variation was observed between micropropagted plantlets and mother plants.

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