IOSR Journal of Biotechnology and Biochemistry (IOSR-JBB)
ISSN: 2455-264X, Volume 1, Issue 3 (Mar. – Apr. 2015), PP 10-14
www.iosrjournals.org

RAPD Analysis Of Rapidly Multiplied In Vitro Plantlets of Anthurium Andreanum Bicolour Var Agnihotri

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Abstract: *The seeds of Anthurium andreanum bicolour var agnihotri were made to germinate in Murashige and Skoog medium. A single good plantlet developed was further taken as explants and cultured in Murashige and Skoog medium supplemented with 2 mg/L of 6-Benzylaminopurine and 0.5 mg/L of naphthaleneacetic acid hormone concentrations. After 30 days the plantlets were transferred to fresh Murashige and Skoog medium with increases in hormone concentration with 4mg/L of 6-benzylaminopurine and 2mg/L- of naphthaleneacetic acid. The proliferated plantlets were maintained in this medium for 90 days. The concentration proved excellent proliferation of shoots in the plantlets. The multiple shoots obtained were transferred to four different media. Murashige and Skoog without hormones, Murashige and Skoog medium with hormone concentration of 4 mg/L 6-benzylaminopurine and 2mg/L- naphthaleneacetic acid, Scheck and Hildebrandt and Hildebrandt et al media was selected for and the plantlets was subcultured for ten cycles. Among these Scheck and Hildebrandt medium showed good response of multiplication. It is noted that the multiplications of shoots that were continued in the Murashige and Skoog medium supplemented with 4 mg/L of 6-benzylaminopurine and 2mg/L of naphthaleneacetic acid for 10 cycles showed further good proliferations. The plantlets from the four media concentrations and the plantlet chosen as explant were subjected to RAPD analysis to study any genetic variability. The plantlets cultured in the Murashige and Skoog medium supplement with4 mg/L 6-benzylaminopurine and 2mg/L naphthaleneacetic acid indicated good variation in the band pattern, inferring that the plantlets have undergone mutation.*

Keywords- *Anthurium andreanum, RAPD, in vitro.*

I. Introduction

Anthurium andreanum bicolor variety agnihotri with large brightly coloured spathe is one of the many hybrids which are cultivated as ornamental and for cut flowers. It has a combination of blood red and grass green coloured spathe. Though Anthuriums are cultivated through vegetative method, the regeneration capacity was found to be less among some of the hybrids. This property was investigated by earlier workers and they developed regenerative method through tissue culture technique. The present investigation aims at multiplying the plantlets through in vitro techniques and generate variations. The variations that have been generated in plantlets can be studied through molecular techniques. In recent years a lot of attention is given to cost effective in vitro techniques and production of plantlets. Variations through in vitro techniques can give rise to horticulturally important varieties.

The RAPD analysis can be done within single species (Williams et al., 1990; Welsh and McClelland, 1990). Diversity within-population and between-population can be assessed (J M Deragon and B S Landry). This technique also holds an additional advantage of not necessarily knowing the DNA sequences to carry out the analysis. PCR based RAPD markers have been previously used to assess the genetic variations in anthurium plants by measuring the genetic diversity within the different species (P Nowbuth et al 2005).

Variations are needed for differentiating the characters of importance and hence it is necessary to detect and document the amount of variation existing within and between populations. DNA marker based fingerprinting uses small amounts of DNA to distinguish variations between species which in turn provides reliable information on their phylogenetic relationships. These DNA markers are not usually influenced by environmental conditions. This in turn helps in explaining patterns of genetic variation among plant populations and also to identify duplicated accessions within germplasm collections (Mohamad et al., 2009). On comparison to other molecular markers, Random amplified polymorphic DNA (RAPD) is known for its simplicity, speed and relatively low cost to study genetic diversity, (Rafalski and Tingey, 1993).

II. Materials And Methods

The seeds of Anthurium andreanum bicolour var agnihotri were collected and made to germinate in Murashige and Skoog medium. A single good plantlet obtained was further chosen as explant and cultured in Murashige and Skoog medium (Table I) supplemented with 2 mg/L –6-benzylaminopurine and 0.5 mg/L-naphthaleneacetic acid. This was called the mother plant. After 30 days the plantlets were transferred to fresh

Murashige and Skoog medium with increases in hormone concentration with 4 mg/L –6-benzylaminopurine and 2mg/L-naphthaleneacetic acid (Med A). The proliferated plantlets were maintained in this medium for 90 days. The multiple shoots obtained were transferred to four different media. Murashige and Skoog without hormones(Table I) (Med B), Murashige and Skoog medium hormone concentration with 4 mg/L –6-benzylaminopurine and 2mg/L-naphthaleneacetic acid (Med C), Scheck and Hildebrandt (Table II) (Med D)and Hildebrandt et al media(Table III) (Med E) was chosen and planters were maintained for ten cycles.

DNA extractions -DNA was extracted from the fresh leaves of plantlets obtained from the four media compositions and the mother plant. Two grams of leaves were ground in a mortar with liquid nitrogen. 15ml of lysis buffer was added. The tubes were incubated at 65˚C for 1 hour with intermittent mixing. The tubes were Centrifuge at 10000 rpm for 10 minutes to separate out the unlysed cells. Supernatant was transferred to a fresh 30 ml centrifuge tube carefully. Equal volume of Chloroform was added and mixed well. This solution is centrifuged at 10000 rpm for 15 minutes. The aqueous layer was pipetted out into the fresh 30 ml centrifuge tube. Equal volume of Isopropanol and $1/10^{th}$ volume of 3M Sodium acetate was added and mixed well. Left at room temperature to stand for 5-10 minutes and centrifuged at 10000 rpm for 10-15 minutes, the supernatant was discarded. The pellet was washed with 1ml of 70% ethanol. The pellet air dried and suspended in 500 µl of 1X Tris- EDTA buffer. Samples were treated with RNAse, and the DNA was purified with columns and taken for RAPD analysis.

PCR reactions and RAPD analysis- Five random primers were used in the experiment the sequence ID discussed in Table IV. The PCR mix for the experiment is detailed in table V. The PCR reaction cycle is set for 40 cycles. The samples after 40 cycles in the eppendoff thermo cycler was run by gel electrophoresis after loading the samples with gel loading buffers. A mid range DNA ruler is run as control and used for detecting. The run gel is documented and analyzed.

III. Results

The present study was carried out to find out the genetic variability (somaclonal variation) among *in vitro* cultured Anthurium andreanum bicolour var agnihotri with the mother plant. A single good plantlet obtained from seed was chosen as explant (fig -6) and cultured in Murashige and Skoog medium (Table I) supplemented with 2 mg/L of 6-benzylaminopurine and 0.5 mg/L of naphthaleneacetic acid. This was called the mother plant (fig-7). After 30 days the plantlets were transferred to fresh Murashige and Skoog medium with increases in hormone concentration with 4 mg/L –6-benzylaminopurine and 2mg/L- naphthaleneacetic acid (Med A). The proliferated plantlets were maintained in this medium for 90 days (fig-8). The multiple shoots obtained were transferred to four different media. Murashige and Skoog without hormones(Table I)(Med B), Murashige and Skoog medium hormone concentration with 4 mg/L –6-benzylaminopurine and 2mg/L-naphthaleneacetic acid (Med C), Scheck and Hildebrandt (Med D)(Table II) and Hildebrandt et al media(Table III)(Med E) and maintained for ten cycles (fig-9). Scheck and Hildebrandt medium showed good response of multiplication. It is noted that the multiplications of shoots that were continued in the Murashige and Skoog medium supplemented with 4 mg/L of 6-benzylaminopurine and 2mg/L of naphthaleneacetic acid for 10 cycles showed further good proliferation.

The PCR analysis on the gel are labeled as Mother, Med A,B,C,D,E for the lanes as the media used to obtained these plants. The RAPD analysis of in vitro cultured Anthurium andraeanum bicolour var agnihotri using OPA 02 (fig 1) primer showed homology in bands proving the genetic identity of the mother plant with that of in vitro cultured plantlets. However the primers opc-07(fig2) showed a unique difference at the band size of 600bp compared to the mother plant. Similarly the primer opb-10 (fig 3) showed unique band pattern at 650bp and opd-02(fig4) showed unique band pattern at 1.4kb.The primer opc-06(fig 5) showed complete difference in band patterns which was unique to the rest of the primers analyzed.

IV. Conclusion

The following factors may be responsible for the genetic variability among the in vitro cultured plantlets. The explants (seed) culture conditions, media composition, concentration of growth regulators. The plantlets might have under gone mutation due to the presence of growth regulators (6-benzylaminopurine and naphthaleneacetic acid) and also for the number of cycles they were cultured (10-12cycles). The plantlets cultured in the Murashige and Skoog medium supplement with4 mg/L 6-benzylaminopurine and 2mg/L-naphthaleneacetic acid indicated good variation in the band pattern, inferring that the plantlets have undergone mutation.

Such genetic variation among the ornamental plants were common during in vitro studies and may lead to the development of a new clone a variety of plant which is useful for commercial exploitation, In Anthurium any morphological characters such as changes in the leaf morphology or spadix can be considered as a new variety of ornamental plant.

Tables

Table I- Murashige and Skoog Media	
Macronutrients	**g\L**
Ammonium nitrate NH_4NO_3	650
Potassium nitrate KNO_3	1900
Magnesium sulphate $MgSO_4.7H_2O$	370
Potassium Dihydrogen Orthophosphate KH_2PO_4	170
Calcium chloride $CaCl_2.2H_2O$	440
Minor salts	
Potassium Iodide KI	0.83
Boric acid H_3BO_3	6.2
Manganese Sulphate $MnSO_4.4H_2O$	22.3
Zinc Sulphate $ZnSO_4.7H_2O$	8.6
Sodium molybdate $Na_2MoO_4.2H_2O$	0.25
Cupric sulphate $CuSO_4.5H_2O$	0.025
Cobaltous chloride $CoCl_2.6H_2O$	0.025
Organic nutrients	
Inositol $C_6H_{12}O_6$	00
Thiamine HCl $C_{12}H_{17}ClN_4OS.HCl$	0.5
Glycine $C_2H_5NO_2$	2.5
Agar	8 g\L
Sucrose $C_6H_{22}O_{11}$	30g\L
Activated charcoal	2 g\L

Table II- Schenk and Hildebrandt Media	
Macronutrients	**mg\L**
Magnesium sulphate $MgSO_4.7H_2O$	400
Potassium nitrate KNO_3	2500
Ammonium phosphate monobasic $NH_4H_2PO_4$	300
Calcium chloride $CaCl_2.2H_2O$	200
Micronutrients	
Manganese Sulphate $MnSO_4.4H_2O$	10
Zinc Sulphate $ZnSO_4.7H_2O$	1.0
Cupric sulphate $CuSO_4.5H_2O$	0.2
Cobaltous chloride $CoCl_2.6H_2O$	0.1
Boric acid H_3BO_3	5
Sodium molybdate $Na_2MoO_4.2H_2O$	0.1
Ammonium phosphate monobasic $NH_4H_2PO_4$	300
Potassium Iodide KI	1.0
Chelating agent	
Ferrous sulphate $FeSO_4.7H_2O$	15
Sodium EDTA $Na_2.EDTA.2H_2O$ ($C_{10}H_{14}N_2O_8Na_2.2H_2O$)	20
Organic nutrients	
Inositol $C_6H_{12}O_6$	1000
Nicotinic acid or niacin (vitamin B3) $C_6H_5NO_2$	0.5
Pyridoxine HCl (vitamin B6) $C_8H_{11}NO_3.HCl$	0.5
Thiamine HCl $C_{12}H_{17}ClN_4OS.HCl$	5
Sucrose $C_6H_{22}O_{11}$	8 g\L
Agar	20 g\L

Table III- Hildebrandt et al Media	
Macronutrients	**mg\L**
Magnesium sulphate $MgSO_4.7H_20$	180
Di Sodium sulphate Na_2SO_4	800
Potassium chloride KCl	65
Potassium nitrate KNO_3	80
Sodium dihydrogen ortho phosphate $NaH_2PO_4.H_2O$	33
Calcium nitrate $Ca(NO_3)_2.4H_20$	400
Micronutrients	
Manganese Sulphate $MnSO_4.4H_2O$	4.5
Zinc Sulphate $ZnSO_4.7H_2O$	6
Boric acid H_3BO_3	0.38
Potassium Iodide KI	3
Chelating Agents	
Ferric Tartarate $Fe_2(C_4H_4O_6)_3$	40
Organic nutrients	
Thiamine HCl $C_{12}H_{17}ClN_4OS.HCl$	0.1
Glycine $C_2H_5NO_2$	3
Sucrose	20g\L
Agar	8 g\L

Table IV-primer sequence		
Sl.no	Primers	sequence
1.	OPA-02	TGCCGAGCTG
2.	OPB-10	CTGCTGGGAC
3.	OPD-02	GGACCCAACC
4.	OPC-06	GAACGGACTC
5.	OPC-07	GTCCCGACGA

Table V-PCR constituents		
	Mastermix	
Components	1X	30X
D.D.H20	17ul	510ul
2X PCR Master MIX	20 µl	600µl
*Random Primer	1µl	30µl
Template	2ul	60ul
Total Volume	40 µl	1200µl

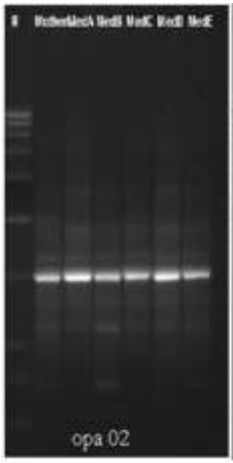
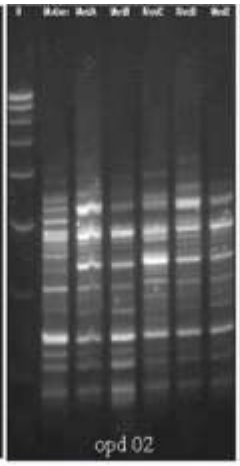


Fig-1



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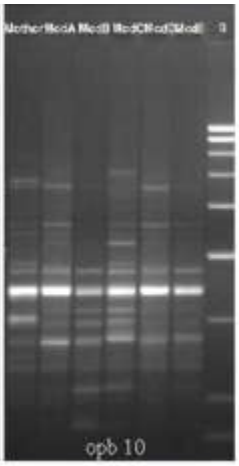

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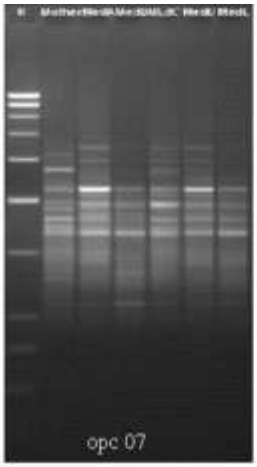

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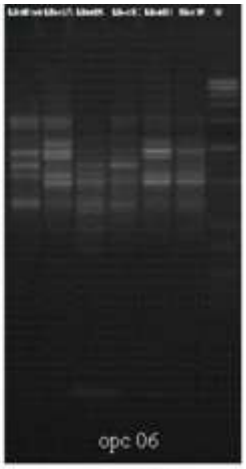

Fig-5

Fig 6

Fig 7

Fig 8

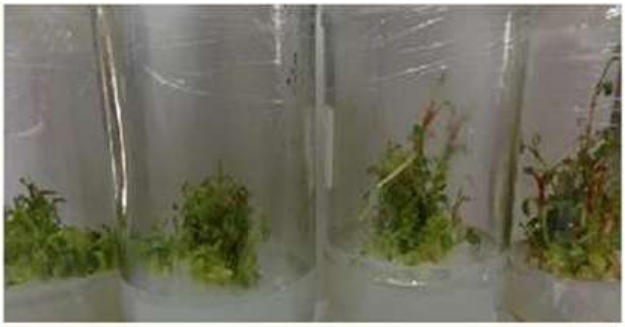

Fig 9

Fig 1-PCR products from opa-02, Lane 1-DNA ruler, Lane 2-Mother plant, Lane 3 to 7-plantlets multiplied in Media composition A to E.
Fig 2-PCR products from opd-02, Lane 1-DNA ruler, Lane 2-Mother plant, Lane 3 to 7-plantlets multiplied in Media composition A to E.
Fig 3-PCR products from opb-10, Lane 1-Mother plant, Lane 2 to 6-plantlets multiplied in Media composition A to E, Lane 7- DNA ruler.
Fig 4-PCR products from opc-07, Lane 1-DNA ruler, Lane 2-Mother plant, Lane 3 to 7-plantlets multiplied in Media composition A to E.
Fig 5-PCR products from opc-06, Lane 1-Mother plant, Lane 2 to 6-plantlets multiplied in Media composition A to E, Lane 7- DNA ruler.
Fig 6- Proliferation of callus from seed.
Fig 7- Plantlet development from callus.
Fig 8 –Plantlets maintained in Murashige and Skoog medium with increases in hormone concentration with 4 mg/L –6-benzylaminopurine and 2mg/L - naphthaleneacetic acid (Med A) for 90 days.
Fig 9-Plantlets proliferated in Med B, C, D, E.

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