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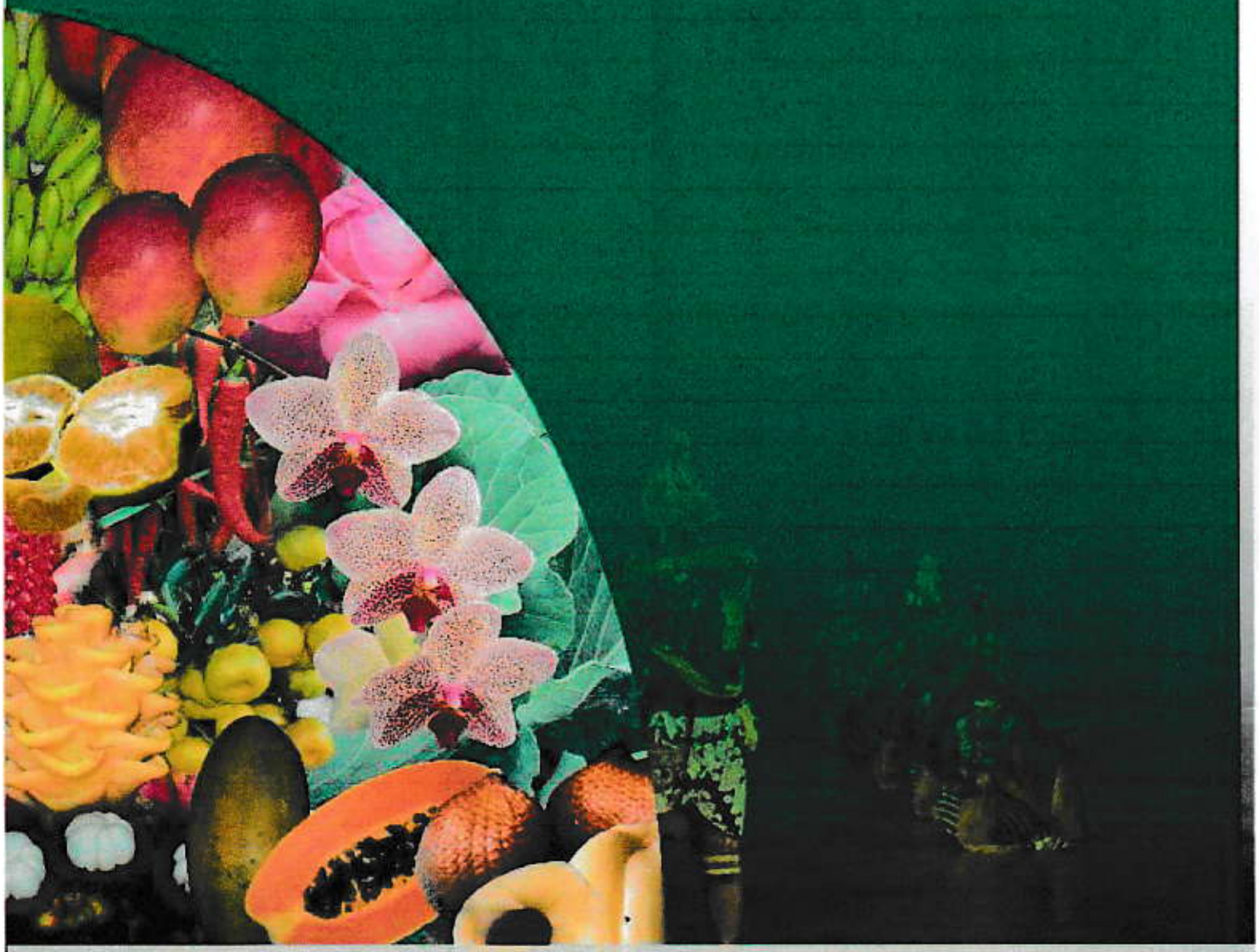

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Optimization of PCR for Detection of Somaclonal Variation in Mangosteen (*Garcinia mangostana* L.) *In Vitro*

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ABSTRACT. Mangosteen *in vitro* plantlets derived from seed have been produced, however an investigation on the somaclonal variation among these plantlets has not been reported before. Detection of somaclonal variation among mangosteen plantlets using Randomly Amplified Polymorphic DNA marker requires the amplification of any DNA segment using short oligonucleotide primers of arbitrary nucleotide sequence and polymerase chain reaction procedures. The objectives of this study are to select primers that produce reproducible polymorphic bands and to optimize each polymerase chain reaction component. A total of 29 primers were screened and 21 of them amplified polymorphic bands. Of the 151 bands obtained, 102 were polymorphic bands and 49 were monomorphic bands ranging between 182 bp – 3320 bp. The percentage of monomorphic bands ranged between 0% - 77.8% whereas the polymorphic bands were between 22.2% - 100% for all primers tested. The highest number of scorable bands was produced using primer AP-20 while the lowest was by OPA-7. The highest percentage of polymorphic bands (100%) was generated by AB-16 whilst the lowest percentage (22.2%) was produced by OPA-5. The optimized of each PCR component for Randomly Amplified Polymorphic DNA is $MgCl_2$ 2 mM, Primer 4 µM, dNTP-mix 0.4 mM, Taq polymerase 1.5U/µL, DNA template 40 ng/ µL. Amplification reaction was run for 40 cycles following the cycle program i.e. initial denaturation at 94°C for 5 minutes, denaturation at 94°C for 30 secons, annealing at 37-38°C for 1 minute, extension at 74°C for 2 minutes and final extension at 72°C for 5 minutes.

Keywords: Mangosteen; *In Vitro*; PCR

Mass propagation of mangosteen through organogenesis was carried out by several researchers (Goh 1990, Te-chato 1995, Normah *et al.* 1995, Huang *et al.* 2000). Direct organogenesis derived from vegetative explants should produce plantlets with uniform genetic traits. However, the concentration of growth regulators used in media, length of culture maintained *in vitro* and frequent subcultures may induce genetic variations in the cultures. This term is defined as somaclonal variation (Larkin & Scrowcoft 1981).

Detection of somaclonal variation was carried out using RAPD. RAPD is a dominant marker system which is defined as the amplification of any DNA segment using short oligodeoxynucleotide primers of arbitrary nucleotide sequence (amplifiers) and polymerase chain reaction procedures (Welsh & McClelland 1990, Devos & Gale 1992, Kahl 2001). PCR is an *in vitro* enzymatic amplification of specific DNA sequences involving DNA denaturation, primer annealing and primer extension (Foolad *et al.* 1995). PCR is characterized by its high speed, selectivity and sensitivity (Weising *et al.* 1995). PCR presents a simple and rapid method of analyzing genetic variation at DNA level, within and between populations (Innis *et al.* 1990).

The specificity of PCR is influenced by several parameters such as the temperature profile of the thermocycler, the annealing temperature, the activity and amount of the polymerase,

concentrations of primers, template DNA and Mg^{2+}. The annealing temperature is usually chosen as high as possible to prevent unspecific amplification (Weising *et al.* 1995). In this study the optimization of PCR parameters was carried out to obtain specific amplification of DNA. The optimized PCR parameters will be used to amplify DNA samples of mangosteen using RAPD marker.

MATERIAL AND METHOD

Plant Materials, Sample Preparation and Primer Tested

Leaf samples were collected from three plants in the mangosteen orchard of UPM Serdang, Malaysia (coded as S1, S2 and S3), one of them is the mother plant which was the source of seeds for the *in vitro* explants (S3), four samples from seedlings collection of the Department

Table 1. Coding of mangosteen leaf samples for assessing genetic variability

Leaf sample code	Treatment	Source of collection
S1	-	Serdang
S2	-	Serdang
S3	-	Serdang
C5	BAP 1 mg/L + NAA 0.1 mg/L	*In vitro*
C6	BAP 3 mg/L + NAA 0.1 mg/L	*In vitro*
C7	BAP 5 mg/L + NAA 0.1 mg/L	*In vitro*
C8	BAP 1 mg/L + NAA 0.5 mg/L	*In vitro*
C9	BAP 3 mg/L + NAA 0.5 mg/L	*In vitro*
C10	BAP 5 mg/L + NAA 0.5mg/L	*In vitro*
C11	BAP 1 mg/L + NAA 1 mg/L	*In vitro*
C12	BAP 3 mg/L + NAA 1 mg/	*In vitro*
C13	BAP 5 mg/L +NAA 1 mg/L	*In vitro*
C14	BAP 0 mg/L + NAA 0 mg/L (MS0)	*In vitro*
C15	BAP 1 mg/L	*In vitro*
C16	BAP 3 mg/L	*In vitro*
C17	BAP 5 mg/L	*In vitro*
P1	-	Pahang
P2	-	Pahang
P3	-	Pahang
P4	-	Pahang
Z1	Soil	Acclimatized
Z2	Soil	Acclimatized
Z3	Sand	Acclimatized
Z4	Peat	Acclimatized
Z5	Soil/Peat (1/1)	Acclimatized
Z6	Sand/Peat (1/1)	Acclimatized

of Agriculture Bukit Goh, Kuantan, Pahang, Malaysia (coded as P1, P2, P3 and P4), 13 leaf samples from the shoots cultured *in vitro* as shown in Table 1, 6 samples from 2 months-acclimatized plantlets (coded as Z1, Z2, Z3, Z4, Z5 and Z6).

Thus total leaf samples collected for DNA isolation, amplification, and analysis using RAPD were 26 accessions.

Leaves were collected by washing it under tap water for 5 minutes. The leaves were sterilized in 5% Clorox solution for 1 minute and rinsed in sterile distilled water three times. Leaves were dried by putting them in between paper cloths. The leaf lamina was separated off from the vein and cut into small pieces. These excised pieces were used for DNA isolation or frozen in liquid nitrogen and stored at -80°C until extraction.

Table 2. A total of 29 RAPD primers used in preliminary primer screening

Primer Name	Sequence 5' To 3'	Melting temperature (°C)
AB-16	CCCGGATGGT	43.6
AE-11	AAGACCGGGA	39.5
AO-12	TCCCGGTCTC	43.6
AP-20	CCCGGATACA	39.5
AV-03	TTTCGGGGAG	39.5
BB-18	CAACCGGTCT	39.5
PO-5	CCCCGGTAAC	43.6
W-15	ACACCGGAAC	39.5
OPB-14	TCCGCTCTGG	43.6
OPA-01	CAGGCCCTTC	43.6
OPA-02	TGCCGAGCTG	43.6
OPA-03	AGTCAGCCAC	39.5
OPA-04	AATCGGGCTG	39.5
OPA-05	AGGGGTCTTG	39.5
OPA-06	GGTCCCTGAC	43.6
OPA-07	GAAACGGGTG	39.5
OPA-08	GTGACGTAGG	39.5
OPA-09	GGGTAACGCC	43.6
OPA-10	GTGATCGCAG	39.5
OPA-11	CAATCGCCGT	39.5
OPA-12	TCGGCGATAG	39.5
OPA-13	CAGCACCCAC	43.6
OPA-14	TCTGTGCTGG	39.5
OPA-15	TTCCGAACCC	39.5
OPA-16	AGCCAGCGAA	39.5
OPA-17	GACCGCTTGT	39.5
OPA-18	AGGTGACCGT	39.5
OPA-19	CAAACGTCGG	39.5
OPA-20	GTTGCGATCC	39.5

A total of 29 primers from Operon Technology (Alameda, CA) (Table 2) were used for preliminary amplification and primer selection. Primers that produced reproducible polymorphic bands were selected. Nine primers tested were referenced by Ramage *et al.* (2004). They were AB-16, AE-11, AO-12, AP-20, AV-30, BB-18, PO-5, W-15 and OPB-14 which were all able to detect genetic variability among mangosteen accessions *in vivo*. Meanwhile, the other primers such as OPA1 – OPA20 were randomly chosen for screening.

DNA Isolation and Quantification

Genomic DNA was isolated from leaf samples using modified CTAB method (Ramage *et al.*, 2004). 1.5 - 3 gram of frozen leaf sample was ground in liquid nitrogen with 1 mol of pre-warmed (65°C) CTAB. The DNA sample was then extracted with 15 mL CIA (24:1) twice and precipitated by adding 2/3 volume of isopropanol. The supernatant was decanted and the pellet washed by 10 mL washing buffer. Subsequenty, the pellet was dried and dissolved with 500 µL of TE buffer pH 8. DNA solution was transferred into 1.5 mL tube and added with 1 µL of RNAse A to remove RNA. DNA was then diluted in TE buffer pH 8.

A DNA quality check was performed in order to ensure that the DNA was not degraded. Eight µL of genomic DNA from each sample was mixed with 2 µL loading dye and run through a 1% agarose gel in electrophoresis machine with 80 V for 35 minutes. The gels were documented using SynGene Gel Doc system.

Each genomic DNA sample was checked for the level of purity and concentration using a nanodrops spectrophotometer ND 1000 V352. Then, the DNA was diluted with sterilized distilled water to prepare 10 ng/µL of DNA concentration. Diluted DNA was used for PCR amplification or stored at -20°C until use.

Table 3. PCR Thermocycler programs

Step	Temperature (°C)	Running Time
Initial denaturation	94	5 minutes
Denaturation	94	30 seconds
Annealing	37 – 38	1 minute
Extension	72	2 minutes
Final extension	72	5 minutes

Polymerase Chain Reaction (PCR) Optimization and Primer Screening

The protocol for RAPD analysis was adapted from that of Williams *et al.* (1990). Optimization of each PCR component and the annealing temperature was carried out before running all DNA samples with primers in PCR Biometra T Personal thermocycler machine. Optimizing the annealing temperatures for each primer was carried out using PCR Biometra T Gradient thermocycler machine.

Amplification reaction was carried out following the cycle program in Table 3. Amplification was run for 40 cycles. Amplification products were analyzed by electrophoresis in 1.5% agarose gel. The gel was run for 90 minutes at 70 V and visualized by staining with ethidium bromide.

A total of 29 primers (Table 2) were screened and optimized to determine those giving scorable bands based on the different ratio of PCR components and different annealing temperatures. Then, subsets of polymorphic primers were chosen to assess the fidelity and diversity of all accessions. Amplification of each primer for all samples was repeated three times.

RESULT AND DISCUSSION

DNA Isolation and Quantification

Isolation of genomic DNA may be inhibited by several factors such as partial or total DNA degradation due to the presence of endogenous nucleases and co-isolation of highly viscous

Table 4. DNA ratio and concentration of 26 accessions of mangosteen from *in vitro* shoots, acclimatized plantlets and different location in Malaysia

Accession Code	Collection	*In vitro* treatment (mg/L)	Ratio of DNA (260/280)	Concentration of DNA (µg/mL)
S1	Serdang	-	1.98	397.9
S2	Serdang	-	2.00	352.3
S3	Serdang	-	2.00	170.4
C5	*In vitro*	BAP 1 + NAA 0.1	2.00	64.0
C6	*In vitro*	BAP 3 + NAA 0.1	1.93	216.5
C7	*In vitro*	BAP 5 + NAA 0.1	2.00	428.0
C8	*In vitro*	BAP 1 + NAA 0.5	1.97	206.5
C9	*In vitro*	BAP 3 + NAA 0.5	2.00	104.9
C10	*In vitro*	BAP 5 + NAA 0.5	1.99	317.5
C11	*In vitro*	BAP 1 + NAA 1	1.98	135.0
C12	*In vitro*	BAP 3 + NAA 1	1.88	171.0
C13	*In vitro*	BAP 5 + NAA 1	1.96	242.5
C14	*In vitro*	MS0	1.98	200.3
C15	*In vitro*	BAP 1	1.98	378.9
C16	*In vitro*	BAP 3	1.94	325.8
C17	*In vitro*	BAP 5	1.86	75.0
P1	Pahang	-	1.90	242.4
P2	Pahang	-	1.94	149.5
P3	Pahang	-	2.00	54.0
P4	Pahang	-	2.00	219.5
Z1	Acclimatized	Soil	1.97	294.9
Z2	Acclimatized	Soil	2.00	323.6
Z3	Acclimatized	Sand	2.00	255.0
Z4	Acclimatized	Peat	1.97	314.1
Z5	Acclimatized	Soil/Peat (1/1)	1.97	107.6
Z6	Acclimatized	Sand/Peat (1/1)	2.00	137.7

Table 5. Optimization of PCR conditions for RAPD analysis technique in mangosteen

PCR component	Stock conc.	Tested range	Volume range	Optimum volume	Final conc (1x)
$MgCl_2$	25 mM	1.0 – 5.0 mM	1.0 – 5.0 µL	2.0 µL	2 mM
Primer	100 µM	0.1 – 8.0 µM	0.025 – 2.0 µL	1.0 µL	4 µM
dNTP-mix	10 mM	0.2 – 0.4 mM	0.5 – 1.0 µL	1.0 µL	0.4 mM
Taq-polymerase	5 U/µL	0.5 - 1.5 U	0.1 – 0.3 µL	0.3 µL	1.5 U
DNA template	10ng/ µL	20 – 50 ng	2.0 – 5.0 µL	4.0 µL	40 ng
PCR buffer	10 x			2.5 µL	1 x
ddH20				14.2 µL	
Final volume				25.0 µL	
Annealing temperature	- 5° ≤ melting temperature of primer ≤ + 5°				

Table 6. RAPD primers and number of bands scored for each primer

Primers	Bands scored (no.)	Monomorphic bands (no)	Polymorphic bands (no)	Size of the bigger band (bp)	Size of the smaller band (bp)
AB-16	11	0	11	2719	300
AE-11	4	3	1	1787	600
AO-12	6	2	4	2159	651
AP-20	13	1	12	3320	350
BB-18	7	5	2	1500	750
OPB-14	6	2	4	2201	779
PO-5	7	1	6	2324	300
OPA-1	4	1	3	2130	1144
OPA-3	8	6	2	1688	490
OPA-5	9	7	2	2491	216
OPA-6	7	1	6	2431	513
OPA-7	3	1	2	900	550
OPA-8	6	1	5	2900	318
OPA-9	9	3	6	1953	600
OPA-11	11	3	8	2253	340
OPA-12	12	1	11	3034	182
OPA-13	4	2	2	1373	490
OPA-16	9	4	5	2659	476
OPA-17	6	2	4	2300	400
OPA-18	5	2	3	1452	550
OPA-19	4	1	3	2927	574

Table 7. RAPD primers and percentage of bands scored for each primer

Primers	Monomorphic bands (%)	Polymorphic bands (%)
AB-16	0.0	100.0
AE-11	75.0	25.0
AO-12	33.4	66.6
AP-20	7.7	92.3
BB-18	71.5	28.5
OPB-14	33.4	66.6
PO-5	14.3	85.7
OPA-1	25.0	75.0
OPA-3	75.0	25.0
OPA-5	77.8	22.2
OPA-6	14.3	85.7
OPA-7	33.4	66.6
OPA-8	16.7	83.3
OPA-9	33.4	66.6
OPA-11	17.3	72.7
OPA-12	8.4	91.6
OPA-13	50.0	50.0
OPA-16	44.5	55.5
OPA-17	33.4	66.6
OPA-18	40.0	60.0
OPA-19	25.0	75.0

polysaccharides (Weising *et al.* 1995). In mangosteen, the difficulty to obtain pure genomic DNA is influenced by polyphenols compounds which contained in leaf samples. Strategies to optimize DNA isolation should be concerned as addition of compounds into the extraction buffer or essential compounds which are protecting DNA from degradation. Pure genomic DNA has an optical density (OD) ratio of 260:280 values of within range of 1.8 and 2.0. In this study, pure genomic DNA was obtained. An optical density (OD) ratio of 260:280 from 26 leaf samples was ranging from 1.86 to 2.00 (Table 4). This result showed that the protocol of DNA isolation could be used to provide pure genomic DNA samples of mangosteen. The result also showed the concentration of DNA samples obtained which is ranging between 54 µg/mL – 428 µg/mL (Table 4).

Polymerase Chain Reaction (PCR) Optimization and Primer Screening

Optimizing RAPD patterns is laborious since many reaction components as well as any part of the PCR program can be changed with quite unpredictable effects. The brand of polymerase and thermal cycler, as well as annealing temperature and primer, were found to have a major impact on banding pattern quality (Weising *et al.* 1995). In this study, each PCR component was optimized following the standard range for RAPD (Table 5). For each primer, the effect of various amount of Mg^{2+}, Taq polymerase, dNTP and DNA template in a PCR reaction were tested for different annealing temperature using two samples of DNA and control DNA. The final concentration of each component was chosen based on the banding pattern quality which produced from PCR reaction. The concentration of $MgCl_2$ is 2 mM, primer for 4 µM, dNTP-mix for 0.4 mM, taq-polymerase for 1.5 U/µL, DNA template for 40 ng/µL and PCR buffer for 1x as shown on table 5. The annealing temperature was ranging between 37 – 38°C.

The optimization was carried out to choose the optimum primer for PCR program. A total of 29 primers were screened and 21 of them amplified polymorphic bands. Of the 151 bands obtained, 102 were polymorphic bands and 49 were monomorphic bands ranging between 182 bp – 3320 bp as shown in Table 6. The percentage of monomorphic bands ranged between 0% - 77.8% whereas the polymorphic bands were between 22.2 % - 100 % for all primers tested (Table 7).

The highest number of scorable bands was produced using primer AP-20 while the lowest was by OPA-7 (Table 6). The highest percentage of polymorphic bands (100%) was generated by AB-16 whilst the lowest percentage (22.2%) was produced by OPA-5 (Table 7). These results were in agreement with Ramage *et al.* (2004) who reported AP-20 and AB-16 were among the primers that revealed polymorphic bands among mangosteen accessions using RAF.

CONCLUSION

In this study, pure genomic DNA was obtained. An optical density (OD) ratio of 260:280 from 26 leaf samples was ranging from 1.86 to 2.00. The optimized of each PCR component for Randomly Amplified Polymorphic DNA is $MgCl_2$ 2 mM, Primer 4 µM, dNTP-mix 0.4 mM, Taq polymerase 1.5U/µL, DNA template 40 ng/ µL. Amplification reaction was run for 40 cycles following the cycle program i.e. initial denaturation at 94°C for 5 minutes, denaturation at 94°C for 30 secons, annealing at 37-38°C for 1 minute, extension at 74°C for 2 minutes and final extension at 72°C for 5 minutes.

REFERENCES

1. Devos, KM & Gale, MD 1992, 'The use of random amplified polymorphic DNA markers in wheat', *Theory Applied Genetic,* no. 84, pp. 567-72.
2. Goh, HKL, Rao, AN & Loh, CS 1990, 'Direct shoot bud formation from leaf explants of seedlings and mature mangosteen (*Garcinia mangostana* L.) trees', *Plant Science,* no. 68, pp. 113-21.
3. Huang, L, Huang, B, Wang, CH, Kuo, C & Murashige, T 2000, 'Developing an improved in vitro propagation system for slow-growing species using *Garcinia mangostana* L. (Mangosteen)', *In Vitro Cellular and Developmental Biology,* no. 36, pp. 501-04.
4. Foolad, MR, Arulsekar, S & Rodriguez, RL 1995, 'Application of polymerase chain reaction (PCR) to plant genome analysis', In Gamborg, OL & Phillips, GC (eds.), Plant cell, tissue and organ culture fundamental methods, Springer-Verlag, Berlin Heidelberg.
5. Innis, MA, Gelfand, DH, Sninsky, JJ & White, TJ 1990, *PCR Protocols: A Guide to Methods and Applications*, Academic Press. San Diego. pp. 482.
6. Larkin, P & Scowcroft, WR 1981, 'Somaclonal variation, a novel source of variability from cell cultures for plant improvement', *Theoretical of Applied Genetic,* no. 60, pp. 197-214.
7. Lim, AL 1984, 'The embryology of *Garcinia mangostana* L (Clusiaceae)', *The Garden's Bulletin*, Singapore, no. 37, pp. 93-103.
8. Medhi, K, Purohit, BP, Gogoi, AJ, Saikia, SP, Kanjilal, PB & Bhau, BS 2009, Molecular marker based genetic diversity analysis of two economically important plants (Zanthoxylum hamiltonianum and Nepenthes khasiana) of North-East India', In Marngar, D & Jyrwa, S (eds.), *Biodiversity herbal medicine,* Synod College and Akansha Publishing House, New Delhi, India, pp. 241.
9. Normah, MN, Nor-Azza, AB & Aliudin, R 1995, 'Factors affecting *in vitro* shoot proliferation and *ex vitro* establishment of mangosteen', *Plant Cell, Tissue and Organ Culture,* no. 43, pp. 291-4.
10. Ramage, CM, Sando, L, Peace, CP, Carrol, BJ & Drew, RA 2004, 'Genetic diversity revealed in the apomictic fruit species *Garcinia mangostana* L. (mangosteen)', *Euphytica,* no. 136, pp. 1-10.
11. Te-chato, S, Lim, M & Suranilpong, P 1995, Plantlet formation from leaf-derived embryogenic callus of mangosteen. Songklanarin, *Journal of Science and Technology,* no. 17, vol. 2, pp. 129-35.
12. Weising, K, Nybom, H, Wolff, K & Meyer, W 1995, *DNA Fingerprinting in Plants and Fungi*, CRC Press Inc. Boca Raton, Florida, pp. 322.
13. Welsh, J & McClelland, M 1990, 'Fingerprinting genomes using PCR with arbitrary primers', *Nucleic Acids Research*, no. 18, pp. 7213-8.
14. Williams, JGK, Kubelik, AR, Livak, KJ, Rafalski, JA & Tingey, SV 1990, 'DNA polymorphisms amplified by arbitrary primers are useful as genetic markers', *Nucleid Acid Research,* vol. 18, no. 22, pp. 6531-5.