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OPTIMIZATION OF RAPD METHOD AND ITS APPLICATION FOR THE ANALYSIS OF GENETIC VARIABILITY IN CULTIVATED AND WILD INDIAN SESAME

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ABSTRACT

The importance of the oilseed crop sesame in agro-based industry has challenged the improvement of its population selection, as it takes longer time by traditional methods. The selection period can be reduced by the application of Random Amplified Polymorphic DNA (RAPD) technique. There are factors influencing the results of RAPD, such as Concentration of Mg^{2+}, dNTPs, DNA template, *Taq* polymerase, Annealing temperature etc. These conditions for RAPD were optimized for the molecular characterization of some of the cultivated and wild sesame (*Sesamum indicum*, *S. mulayanum*). The influence of various factors like concentration of Mg^{2+}, DNA template, primer, *Taq* polymerase, dNTPs, phases and the number of PCR cycles were determined and optimized for Random Amplified Polymorphic DNA (RAPD) analysis of sesame. A combination of Mg^{2+} at 3.0 mM, primer at 0.8 µM (Operon Biotechnologies), dNTPs at 0.12 µM (SibEnzyme), genomic DNA at 50 ng, *Taq* polymerase 1.5 U (SibEnzyme) in 25 µl reaction mixture with 45 PCR cycles was best for RAPD amplification. Application of these optimized conditions for RAPD analysis for genetic diversity assessment of *Sesamum spp.* showed enormous diversity among cultivated and wild sesame accessions. This study provides an enriched RAPD protocol suitable for molecular analysis of sesame and helpful for breeding.

KEYWORDS: *Sesamum indicum*, *S. mulayanum*, genetic diversity, Random Amplified Polymorphic DNA, Optimized conditions

Sesame (*Sesamum sp.*, Family: Pedaliaceae) is one of the oldest cultivated oilseed plants in the world according to archaeological records (Nayar, 1984). As per FAOSTAT (2011) India was world's second largest producers of sesame in 2009. Both *Sesamum indicum* and *S. mulayanum* are annual, indeterminate plants with a diploid chromosome number of 2n = 26 (IPGRI and NBPGR 2004). Sesame oil comprising 50 % of the dry seed weight has been preferentially consumed in oriental food because of its distinctive flavor and the stabilizing antioxidant properties. It is used as active ingredient in antiseptics, viricides and disinfectants, and is a considerable source of calcium, tryptophan, methionine and many minerals (Anilakumar et al., 2010). High level of polymorphism in sesame has been reported before for its morphology (Bisht et al., 1998). In spite of that, it has been a neglected crop with a low priority in research support. The research on sesame genetic diversity at molecular level has been scarce. Selection of elite varieties using molecular markers is more rapid and can reduce the costs of maintaining a large number of field trials making it more economical than the selection using phenotype analysis (Punia et al., 2009).

Random amplified polymorphic DNA (RAPD), which is a molecular marker that has evolved from polymerase chain reaction (PCR) technology, has become an essential tool for molecular genetic studies (Punia et al., 2009). This method has been used in genetic analysis for identification of varieties, construction of genetic maps etc. There are many factors influencing the results of RAPD, such as Concentration of Mg^{2+}, dNTPs, DNA template, *Taq* polymerase, Annealing temperature etc. The main objective of this study was to develop a RAPD protocol suitable for sesame and PCR thermocycler parameters were decided after preliminary comparison of a number of commonly used protocols (Ostrowska et al., 1998) for sesame. In the present study, attempts were made using altering concentrations of the RAPD reagents for the establishment of optimum conditions. The most suitable PCR thermocycler profile was selected to produce substantial number of clear bands that are useful as genetic markers and genetic diversity among *S. indicum* and *S. mulayanum* were studied.

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MATERIALS AND METHODS

Materials

The seeds of three cultivars of sesame (*Sesemum indicum* L.), TKG-21, SVPR-1 and Krishna were received from National Bureau of Plant Genomic Research (NBPGR), New Delhi. Another local high yielding land race of *S. indicum* (WB-BDM) and one of its wild relative *S. mulayanum* Nair (WB-SM) were collected from Gangetic plain of West Bengal. All accessions were grown in Madhyamgram Experimental Farm of Bose Institute during the period 2006 to 2010 under optimum agronomic conditions.

Isolation of DNA and Measurement of DNA Purity and Quality

Five days old cotyledonary leaves of ten accessions were collected for DNA isolation. The extraction was done by E.Z.N.A. high performance plant DNA extraction kit. RNase was added to remove RNA. The yield of DNA was measured using a UV-Spectrophotometer at 260 nm. The purity of DNA was determined by calculating the ratio of absorbance at 260 nm to that of 280 nm. The DNA concentration was determined by the formula:

DNA concentration = OD 260 × 50 μg/ml × dilution factor.

Stock DNA solution was diluted in TE buffer (pH 8.0) to a final concentration of 50 ng/μl.

PCR Optimization

Arbitrary primer fingerprinting kits (OPA and OPM) were obtained from Operon Biotechnologies Ltd., U.S.A. All two kits contained 20 different sequences of 10 oligonucleotide primers out of which 15 sequences were selected randomly (with the requirement for 60% to 70% G + C content for sequence stability at high temperatures) and were used to check the fidelity of amplification.

Amplification was performed in 25 μl PCR tubes each tube containing 10× PCR buffer (2.5 μl, Mg^{2+} free) of SibEnzyme Ltd. Russia, $MgCl_2$ of Promega Corporation U.S.A. (2.5 mM to 3.5 mM), dNTPs of SibEnzyme Ltd. Russia (0.08 mM to 0.12 mM), primers of Operon Biotchnologies Ltd. U.S.A. (0.4 μM to 0.8 μM), Taq polymerase of SibEnzyme Ltd. Russia (0.5 U to1.5 U), DNA sample (10 ng to 50 ng) and HPLC grade H_2O was added to make the final volume to 25 μl (Table, 1). The optimum conditions were analysed by altering a single factor and keeping the other conditions constant. PCR reaction was carried out in a DNA Thermal Cycler (Labnics 9600 PCR system, U.S.A.). The PCR amplification conditions were optimized as follows: 90ºC, 92ºC and 94ºC were chosen to optimize the initial extended step of denaturation followed by 34 - 44 cycles of denaturation (in 90ºC / 92ºC / 94ºC) for 1 min.; primer annealing at 32ºC / 34ºC / 35ºC / 36ºC, 37ºC / 38ºC for 1 min. and elongation at 72ºC for 2 min., followed by an extended elongation step at 72ºC for 7 min. (Table, 2). This was carried out RAPD analysis in ten different varieties of sesame.

The PCR products were run in 1.8% Agarose gel. Molecular weights of the bands were estimated by using 500 bp Express DNA ladder and 100 bp DNA ladder (Fermentas Laboratories Ltd. U.S.A.) as standards. In case of RAPD after completion of running, the gel was stained by pouring it into a tray containing Ethidium Bromide (10 μl Ethidium Bromide from 10mg/ml stock was added to 100 ml of double distilled water) solution for 30 min. in dark followed by 5 min. thorough washing under running water. The gel was visualized in Gel Documentation instrument.

Band Scoring and Data Analysis

Each amplification product was considered an RAPD marker. Bands were scored across all samples by using Quantity One software. They were recorded using binary system viz. present (1) or absent (0). Molecular weights of the bands were estimated by using 500 bp Express DNA ladder and 100 bp DNA ladder (Fermentas Laboratories Ltd.) as standards. All amplifications were replicated thrice and only reproducible bands were considered for the calculation of Polymorphism percentage as the proportion of polymorphic bands over the total number of bands.

RESULTS AND DISCUSSION

PCR based molecular marker RAPD allows the rapid detection of DNA polymorphism from many individuals or pooled samples in order to avoid erratic amplifications, good quality of genomic DNA free from contaminants and optimization of PCR reaction conditions is a perquisite for developing strategies for crop

improvement programs in future.

Optimization of DNA Template Concentration

In order to determine the optimal concentration of template genomic DNA, the concentrations were varied from 20 ng to 100 ng retaining all other reaction conditions constant. The reactions were replicated thrice to examine the possible influence of DNA concentration on the fidelity of PCR amplifications. The results clearly reveal that only the concentration range of ~50 ng yielded reproducible patterns. Too little template DNA gave variable banding patterns while excess amounts of genomic DNA resulted in non-specific amplifications (Table, 1). Variability at low template concentrations may be as a result of a reduced probability in initiating amplification reactions and reflects the inefficiency of the priming events as reported by Welsh and McClelland (1990).

Optimization of Mg^{2+} Concentration

Three different concentrations of Mg^{2+} as 2.5 mM, 3 mM and 3.5 mM were used to study the influence of Mg^{2+} ions on the efficiency and fidelity of the RAPD amplifications. The results showed that the lane 2 has a good amplification profile where concentration of Mg^{2+} was 3.0 mM and hence considered as optimum (Fig., 1). This clearly demonstrates relationship between Mg^{2+} and DNA concentrations and suggests that the stringency of annealing process is decreased at higher Mg^{2+} concentrations (Table,1). Using suboptimal concentrations of $MgCl_2$ can result in reduced product yield (Zangenberg et al., 1999; Wang et al., 2009).

Optimization of Primer Poncentration

The primer concentrations of 0.4 µM, 0.6 µM and 0.8 µM were tested by retaining all other reaction conditions at constant. The best amplification results were obtained with primer concentration of 0.8 µM as seen in lane 3 (Fig.,2). It is likely that raising the primer concentration will lead to non-specific primer binding and the creation of spurious, undesirable PCR products (Table,1). When the primer concentration is reduced, no amplification may occur or there may be a drop off in band number and intensity. It appears that the ratio of the primer concentration and template DNA concentration is most critical. The titer of the template DNA concentration should be carefully determined against a fixed primer concentration to obtain ideal conditions (Tyler et al., 1997).

Optimization of Taq Polymerase Quantity

Maintaining all the other previously described reaction conditions constant, the best amplification result was obtained with Taq polymerase (SibEnzyme Ltd.) quantity 1.5 U (Table, 1) compared with that of 1 U and 0.5 U, (Fig., 3). Lower concentration resulted in decreased specificity and background (smear) formation upon gel electrophoresis (Harini et al., 2008). The efficiency of the same polymerase can vary significantly depending on the nature of the target sequence, the primer sequences, and the reaction conditions (Tyler et al., 1997).

Optimization of dNTPs Concentration

While the other reaction conditions remained the same, the different dNTPs concentrations of (each) dATP, dGTP, dCTP and TTP (SibEnzyme Ltd) were analysed viz., 0.08 mM, 0.1 mM & 0.12 mM. The ideal amplification results obtained when dNTPs concentration was 0.12 mM as seen in lane 3 (Fig., 4). Amplification reproducibility rate was low in case of reduced concentrations of dNTPs. The intensity of the higher molecular weight bands was significantly greater at the highest concentration (0.12mM) of the experimental range taken in the present case (Table,1). Moreover, higher concentrations of dNTPs were avoided because those increase the error rate of the Taq DNA polymerase activity (Gelfand, 1989).

Optimization of PCR Amplification Cycles

Different number of PCR amplification cycles was tested with 35, 45 and 55 cycles per reaction. Results indicated that 45 cycles performed best for amplification (Table,2). Conclusively, the optimal RAPD reaction conditions of sesame was obtained by using, 1X PCR buffer, Mg^{2+} 3.0 mM; Primer 0.8 µM; DNA template 50 ng; each dNTPs 0.12 mM; Taq polymerase 1.5 U and HPLC grade water to make the final volume to 25 µl (Table,1).

The different PCR parameters, namely temperatures, durations and 'ramping' of denaturation, annealing and extension steps, as well as the number of amplification cycles could be altered to obtain optimal

Table 1: Optimization of RAPD-PCR reaction parameters for *Sesamum indicum* with respect to 25 µl PCR mix

Parameters	Experimental range	Optimum conditions	Inference
Template DNA concentration	10 - 50 ng	~50 ng	Concentrations of about 50 ng of DNA produced intense bands that were easier to record.
Magnesium chloride ($MgCl_2$) concentration	2.5 mM, 3.0 mM & 3.5 mM	3.0 mM	Lower concentrations of $MgCl_2$ gave poor amplification and the stringency of annealing process is decreased at higher Mg^{2+} concentrations.
dNTPs concentration	0.08 mM, 0.1 mM & 0.12 mM	0.12 mM	Amplification reproducibility rate was low in case of reduced concentrations of dNTPs.
Primer concentration	0.4 µM, 0.6 µM & 0.8 µM	0.8 µM	The primer concentration must be high enough to generate proper amplification products as the lower concentrations of primer failed to generate that.
Taq polymerase concentration	0.5 U, 1 U & 1.5 U	1.5 U	Decreased specificity and background smear formations (upon gel electrophoresis) were depicted by Lower concentrations of *Taq* polymerase.

Table 2: Optimization of RAPD-PCR cycle for *Sesamum indicum*

Parameters	Experimental range	Optimum conditions	Inference
Denaturation temperature	90 ºC, 92 ºC, 94 ºC	94 ºC	Poor amplified products were obtained with lower denaturation temperature
Initial denaturation time	2 min., 3 min., 4 min., 5 min.	4 min.	Higher time interval resulted in reduced amplification, loss of Taq polymerase activity & lack of reproducibility
Annealing temperature	32 ºC, 34 ºC, 35 ºC, 36 ºC, 37 ºC, 38 ºC	35 ºC	Non -specific amplifications were found upon using lower / higher annealing temperature (from optimum)
Number of cycles	35 - 55	45 cycles	Poor amplification was observed in reduced number of cycles and background formation was obtained in more than 45 cycles

Table3: List of RAPD Primers revealing ≥ 6o % Polymorphism among the selected accessions

Primer	Sequence	Total Amplified Bands	No. of Polymorphic Bands	Percentage (%) of Polymorphism
OPA -2	5′-TGC CGA GCT G -3′	11	9	81.82
OPA -3	5′-AGT CAG CCA C -3′	11	7	63.64
OPA -4	5′-AAT CGG GCT G -3′	5	3	60
OPA -5	5′-AGG GGT CTT G -3′	10	7	70
OPA -9	5′-GGG TAA CGC C -3′	9	8	88.89
OPM -10	5’ -TCT GGC GCA C -3’	7	5	71.43
OPM -16	5′-GTA ACC AGC C -3′	8	7	87.5
OPM -20	5′-AGG TCT TGG G -3′	7	5	71.43

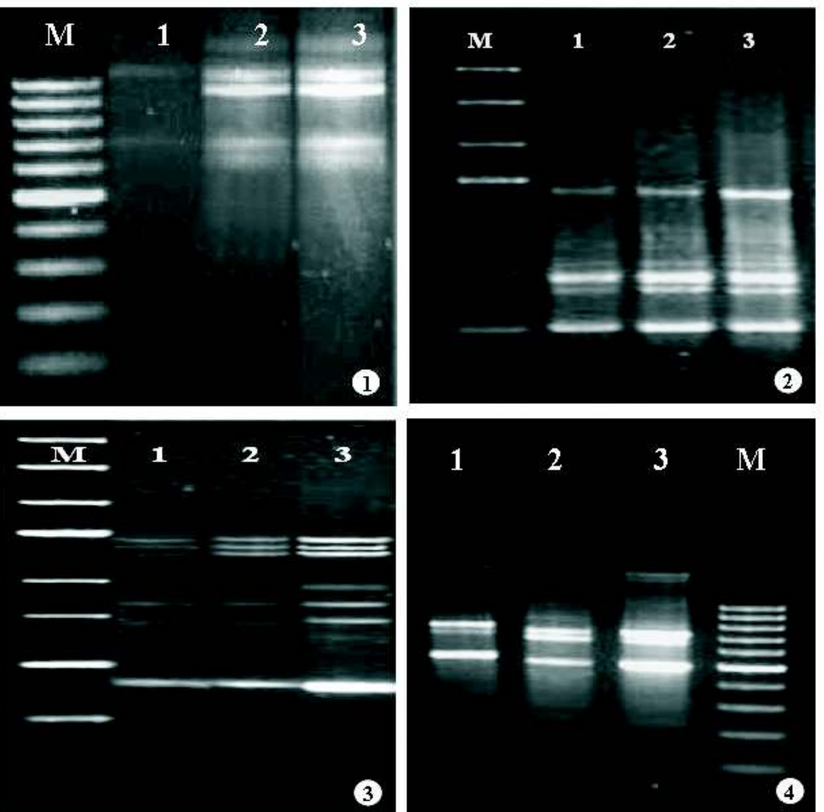

Fig. 1-4: RAPD banding patterns of sesame during optimization,

1) using different Mg2+ concentrations- Lane 1:2.5 mM; Lane 2: 3 mM; Lane 3: 3.5 mM (M: 100 bp DNA ladder, primer OPA-18, var. Krishna).
2) using different primer concentrations- Lane 1: 0.4 M; Lane 2: 0.6 M; Lane 3: 0.8 M (M: 100 bp DNA ladder, primer OPA-3, var. SVPR-1).
3) using different concentrations of *Taq* polymerase. Lane 1: 0.5 U; Lane 2:1 U; Lane 3: 1.5 U (M: 500 bp DNA ladder, primer OPA-9, var. SVPR-1).
4) using different concentrations of dNTOs- Lane 1: 0.08 mM; Lane 2:0.1 mM; Lane 3: 0.12 mM (M: 100 bp DNA marker, primer OPA-2, var. WB-SM).

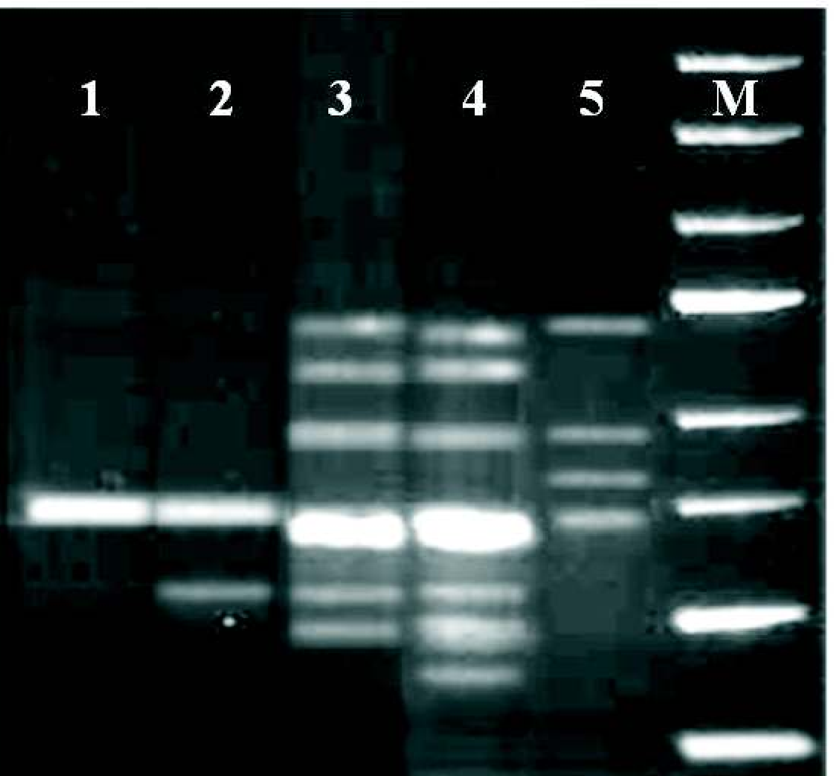

Fig. 5: RAPD profile of the four accessions of *S.indicum* and one *S. mulayanum* obtained with primer OPM-16. Lane M-500 bp molecular mass marker, lanes: 1-TKG-21, 2-SVPR-1,3-Krishna, 4-WB-BDM, 5-WB-

RAPD banding patterns. A notable effect was observed when the standard denaturing temperature of 94ºC with initial pre-denaturation of 4 min. was lowered to 90ºC. A comparison of the pattern reveals major bands produced under a denaturing temperature of 94ºC disappeared and additional minor bands were formed when denaturing was carried out at 90ºC supporting the observations of Punia et al., (2009). The final annealing temperature set at 35°C (Table,2) was also in concomitant as recommended by Operon Technologies. Zangenberg et al., (1999) observed that the lower annealing temperatures often results in overall increase in non-specific amplification while the higher annealing temperature results in more specific amplification. Annealing temperatures for arbitrary short primers mainly range from 34 to 36°C but can clearly be defined with more precision depending on the melting temperature of each primer. Methods have been developed to calculate the optimum annealing temperatures in a PCR reaction (Rychlik et al., 1990). Optimal annealing temperature for RAPD must consequently be determined empirically. Different number of PCR amplification cycles was tested with 35, 45 and 55 cycles per reaction. Results indicated that 45 cycles performed best to obtain amounts of DNA which could be detected by agarose gel electrophoresis.

Band Scoring And Data Analysis

The effectiveness of the RAPD markers in characterization of Sesame germplasm demonstrated here was exploited for analyses of structure and extent of the genetic diversity. Nine primers in the present study resulted unique bands. Primer OPA-2, OPA-4, OPA-5, OPA-9, OPA-18, OPM-10, OPM-16, OPM-20 were able to reveal ≥ 60% polymorphism among the selected accessions. This discrimination was expected as the objective was to find out diversity in minute molecular level and can be useful for obtaining cultivar/ genotype specific profiles as they showed high level of polymorphism among the selected accessions. The reproducibility of RAPD amplification is known to be highly influenced by experimental conditions (Wolff et al., 1993). Conclusively, the optimal RAPD reaction conditions of S. indicum was obtained by using 1X PCR buffer, Mg^{2+} 3.0 mM; Primer 0.8 µM; DNA template 50 ng; each dNTPs 0.12 mM; Taq polymerase 1.5 U and HPLC grade water to make the final volume to 25 µl and the PCR amplification cycles were 45. Under the optimized conditions as standardized above, the RAPD-PCR analysis of sesame was carried out and the results (Fig. 5) showed that the bands amplified were abundant and clear as well as ample quantity of genetic diversity was present in inter and intra specific level. Possibility of achieving improvement in any crop plants depends immensely on the magnitude of genetic variability. The evidences proved that the RAPD assay of sesame was standardized successfully and the results thus obtained after optimization of the conditions for PCR amplification are useful for phylogenetic analysis.

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