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Optimization Protocol of DNA Isolation and PCR in Muskmelon (*Cucumis melo* L.) by RAPD Marker

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The present investigation entitled “Optimization Protocol of DNA isolation and PCR in Muskmelon (*Cucumis melo* L.) by RAPD Marker” was carried out at Department of Plant Biotechnology SDMVM’s College of Agricultural Biotechnology, Georai Tanda, Paithan Road, Aurangabad (M.S.), 431001. Six different commercial verities of muskmelon plant were studied for this research. Standardization protocol of DNA isolation from muskmelon - One method (CTAB) was studied for obtaining good quality DNA from muskmelon. It is showing that when C-TAB method were used good quality of DNA obtained and its quantity 100ng. When C-TAB method used for isolation of DNA from Muskmelon, three extraction buffers were used for optimizing condition for DNA isolation from C-TAB method. Five primers were used for testing polymorphism and monomorphism percentage. Out of five primer A14 primer showed 22.22% polymorphism and remaining primer showed monomorphism. Total primer showed 4.1% polymorphism and 95.10% monomorphism.

Introduction

In India is usually called kharbuja. Melons, *Cucumis melo* L., are important horticultural crops in tropical and subtropical regions, which are also grown extensively in temperate climates. Muskmelon (*Cucumis melo*) is a species of melon that has been developed into many cultivated varieties. Muskmelon is native to Iran, Anatolia, Armenia, and adjacent areas on the west and the east which is believed to be their center of origin and development, with a secondary center including the northwest provinces of India and Afghanistan. Although truly wild forms of *C.*

melo have not been found, several related wild species have been noted in those regions. The muskmelon contains minerals like potassium, sodium and magnesium and vitamin A, B and C. It is also an excellent source of the antioxidant vitamin C. Fruit of muskmelon is an excellent source of Vitamin A, (100 g provides 3382 IU or about 112% of recommended daily levels) one of the highest among cucurbita fruits. Vitamin A is a powerful antioxidant and is essential for healthy vision. It is also required for maintaining healthy mucus membranes and skin. Consumption of natural fruits rich in vitamin A has been known to help protect

from lung and oral cavity cancers. It is also rich in antioxidant flavonoids such as *beta-carotene*, *lutein*, *Zea-xanthin* and *cryptoxanthin*. Potassium is an important component of cell and body fluids and helps control heart rate and blood pressure. It thus offers protection against stroke, and coronary heart diseases. Molecular marker is a gene or DNA sequence with a known location on a chromosome that can be used to identify individuals or species. They are identifiable DNA sequence, found at specific locations of the genome, and transmitted by the standard laws of inheritance from one generation to the next generation. Number of Fruit crop utilization and conservation has attracted global attention. Several of these fruit crop belonging from cucumber family contain exceptionally high amounts of polysaccharides, polyphenols, tannins, hydrocolloids (sugars and carragenans) and other secondary metabolites such as alkaloids, flavonoids phenols, terpenes and quinines which would interfere with the DNA isolation procedures. The problems encountered in the isolation and purification of DNA include degradation of DNA due to endonucleases, isolation of highly viscous polysaccharides, inhibitor compounds like polyphenols and other secondary metabolites which directly or indirectly interfere with the enzymatic reactions. Present investigation was carried out with an objective 1. To standardize protocol for DNA isolation from muskmelon 2) To Optimize PCR protocol for RAPD marker in muskmelon.

Materials and Methods

The present study “Optimization Protocol of DNA isolation and PCR in Muskmelon (*Cucumis melo* L.) by RAPD Marker” was carried out at Department of Plant Biotechnology SDMVM’s College of Agricultural Biotechnology, Georai Tanda, Paithan Road, Aurangabad (M.S.), 431001

during Jan. 2014 to May 2014. Six different commercial of different verities of muskmelon plant were used. Six healthy seeds of each variety were selected and sown in pots. At every two days interval germination was observed. After two weeks, fresh and young leaves were taken out, washed, weighed and crushed in liquid nitrogen to fine powder. Crushing of leaves was done by prechilled pestle and mortar in liquid nitrogen and care was taken to avoid thawing of the material. Before thawing the material was transferred into 50ml centrifuge tube and kept at -20°C. Chemicals used for present study were of good quality (AR-grade) from various agencies.

Extraction of DNA

Reagents used for the DNA isolation

I. Extraction buffer:

1 gm CTAB
3 M (17.532gm) NaCl
50 mM (0.05µl) EDTA
100 mM Tris-Cl
50µl β – mercaptoethanol (added at the time of use).

The extraction buffer was autoclaved before addition of β – mercaptoethanol.

II. Chloroform: Isoamyl alcohol (24:1)

III. 100 % Isopropanol (Ice-cold)

IV. Wash buffer: 70 % ethanol

V. TE buffer (10 mMTris and 1 mM EDTA, pH 8.0) Autoclaved before use.

Reagents used for PCR and gel preparation

Template DNA: 2µl (30ng) from muskmelon seedlings.

RAPD primers

*Taq*DNA polymerase (5 U/ μ l)

10 X *Taq* assay buffer with MgCl₂ (15 mM)

Tris HCl 1 M (3.152 gm)

Boric acid 1M (12.36) gm

EDTA 25 mM (1.86) gm

25 mM MgCl₂

dNTP (10mM)

Sterile distilled water

6X gel loading dye (0.04 % bromophenol blue, 0.04% xylene cyanol FF, 5 % glycerol in water).

Ethidium bromide (0.5 mg/ml)

Agarose

Loading dye preparation

Bromophenol blue, Xylene cylon, glycerol

Buffers and solutions

Different buffers and solutions used along with their composition are given in Table 1.

RAPD-PCR analysis:

PCR Reaction mix (Master Mix)

Procedure for PCR reaction

Sterile micro centrifuge tubes were numbered and placed on PCR tube stand.

At first 2 μ l of DNA was added to each PCR tube followed by master mix.

Then 1.5 μ l of each forward and reverse primer were added in each PCR tube.

The samples were mixed by brief centrifugation to bring down the content of tube.

PCRs were run on the programmable thermal cycler with the following PCR reactions:

Results and Discussion

Standardization protocol of DNA isolation from muskmelon - one method (CTAB) was studied for obtaining good quality DNA from muskmelon. It is showing that when C-TAB method were used good quality of DNA obtained and its quantity 100ng. When C-TAB method used for isolation of DNA from Muskmelon, three extraction buffers were used for optimizing condition for DNA isolation from C-TAB method (Table 2).

From table 3 is showing that when extraction buffer 1 was used for isolation of DNA from leaf sample of muskmelon about 80 ng of DNA were obtained and it was contaminated with RNA and DNA.

In extraction buffer 1 PVP was not used and muskmelon contains secondary metabolite, small quantity of phenolic substance, sticky substance etc. because of this pellet obtained it was contaminated with carbohydrate and protein.

When extraction buffer 2 was used about 90ng of DNA was obtained here PVP was used and concentration of C-TAB also changed up to 1.5 gm DNA obtained was less contaminated compare to Extraction buffer 2.

From table 4 it is showed that fours primers were used for testing polymorphism and monomorphism percentage. Out of five primer A14 primer showed 22.22% polymorphism and remaining primer showed monomorphism. Total primer showed 4.1% polymorphism and 95.10% monomorphism.

Molecular marker is a gene or DNA sequence with a known location on a chromosome that can be used to identify individuals or species (Table 5).

Table.1 Composition of different buffers and solutions

Sr. No.	Buffer and Solution	Composition
Buffers		
1.	Extraction Buffer	1 gm CTAB, 3 M (17.532gm) NaCl, 50 mM(0.05µl) EDTA 100 mM Tris-Cl, 50µl β – mercaptoethanol
2.	10 x TBE	0.9 M Tris base, 0.9 M Boric acid, 0.32 M EDTA
3.	TE Buffer	10 mM Tris HCl (pH 8.0), 1mM Na ₂ EDTA (pH 8.0)
Solutions		
1.	0.5 M EDTA Na ₂	3.72 g EDTA dissolved in distilled water by stirring vigorously and pH was adjusted to 8.0 with NaOH. Total volume was made up to 100 ml. Solution was sterilized by autoclaving.
2.	Ethidium Bromide	1 g Ethidium Bromide dissolved in distilled water and stirred for few hours to ensure that the dye has dissolved and vol. made up to 100 ml. The container was wrapped in aluminum foil and stored at 4°C.
3.	5 M NaCl	1.752 g of NaCl dissolved in distilled water and volume adjusted to 100 ml with distilled water and solution was sterilized by autoclaving.
4.	1 M Tris HCl	0.788 g of Tris base was dissolved in distilled water by stirring vigorously and pH was adjusted to 8.0 by adding concentrated HCl. The volume was adjusted to 100 ml with distilled water and solution was sterilized by autoclaving.

Table.2 Total volume of PCR reaction mix was made to 25µl of which 22.0µl was master and remaining 3µl was the individual DNA of the genotype

Master Mix	1x
10x <i>Taq</i> polymerase assay buffer with MgCl ₂	2.5 µl
MgCl ₂ (25 mM)	1.5 µl
dNTPs (10 mM)	0.25 µl
<i>Taq</i> polymerase (5 U/µl)	0.067 µl
Sterile distilled water	15.68 µl

Table.3 Extraction buffer used for optimizing condition for DNA isolation by using C-TAB method

Sr. No	Method	Extraction Buffer	Quantity DNA obtained
1	C-TAB	Extraction Buffer 1	80ng (contaminated)
2		Extraction Buffer 2	90 ng
3		Extraction Buffer 3	100ng

Table.4 Optimization of the RAPD-PCR reaction parameters for Muskmelon

PCR Parameter	Tested range	Optimum conditions	Remarks
DNA concentration (ng)	5, 10, 40, 50, 75, 100	75 ng	Absence of amplification with lower concentration and presence of smear at higher concentration affected the repeatability.
Magnesium chloride (mM)	1, 2, 3, 4	3 mM	Excess/lower concentration increases the non-specificity and yield of the product.
Deoxynucleotide triphosphates (dNTPs) (mM)	0.1, 0.2, 0.3	0.2 mM	Increased concentration reduces the free Mg ²⁺ , interfering with the enzyme
Primer concentration (μM)	0.1, 0.5, 1	0.5 μM	Lower and higher concentrations lead to absence of amplification and primer dimer formation, respectively.
Taq polymerase (units)	0.1, 0.2, 0.5, 1.0	0.2 U	Lower concentration did not show proper amplification. High concentration showed decreased specificity.
Annealing temperature (°C)/	25, 30, 35, 37, 65 and 70	37°C for 60 s	Higher/lower annealing temperatures (from optimum) results in difference in specificity
Number of cycles	25, 30, 35 and 50	30	Higher/lower cycles (from optimum) effects the amplification

Table.5 Monomorphism and polymorphism % in muskmelon

Sr. No	Primer	Primer Sequence	Band Size (bp)
1)	A04	AATCGGGCTG	300-200
2)	A05	AGGGGTCTTG	300-950
3)	A07	GAAACGGGTG	500
4)	A08	GTGACGTAGG	200-150
5)	A14	AGGGGTCTTG	300-950

Fig.1 RAPD banding pattern of muskmelon

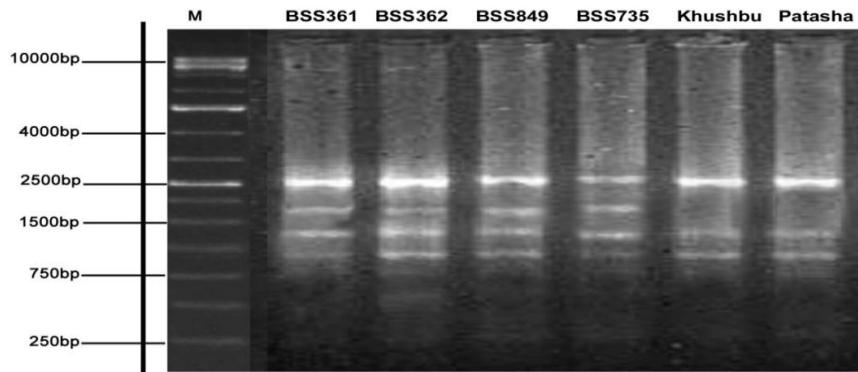


Plate 1 RAPD banding pattern of Muskmelone using A08

They are identifiable DNA sequence, found at specific locations of the genome, and transmitted by the standard laws of inheritance from one generation to the next generation. Five primers were used for testing polymorphism and monomorphism percentage. Out of five primer A14 primer showed 22.22% polymorphism and remaining primer showed monomorphism. Total primer showed 4.1% polymorphism and 95.10% monomorphism (Fig. 1).

From present investigation it is concluded that optimize condition for DNA isolation and PCR analysis required for obtaining accurate result. Pure quality of DNA require for number of studies undertaken in molecular marker technology like phylogenetic study, DNA fingerprinting, linkage mapping etc. Therefore if we have standardized protocol of DNA isolation and PCR analysis then it plays an important role for molecular studies.

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