CHAPTER-III

MATERIALS AND METHODS

3.1 MICROPROPAGATION

3.1.1 Collection of Explants

The seeds of papaya variety i.e. Pusa nanha were collected from IARI, Delhi. The seeds were grown in green house of CPB, Hisar and explants for *in vitro* culture of papaya i.e., shoot tip and nodal segment were collected from these seedlings.

Fig. 3.1: Seedlings of Pusa Nanha in the greenhouse of CPB, Hisar

3.1.2 Glasswares and Chemicals

Glasswares made up of borosilicate were used for the present study and obtained from Borosil India Limited and Corning Glass Company. For culturing experiments the jam bottles were used. The high purity chemicals used in the investigation were of analytical grade and purchased from Hi-Media Limited, Imperial Bioscience and Merck.

3.1.3 Sterilization of glasswares

All the glasswares viz., petridishes, beakers, measuring cylinders, test tubes, flasks, pipettes, jam bottles etc. were washed with liquid detergent followed by washing under running tap water, finally rinsing with double distilled water prior to use. The forceps, scalpel, petridishes and other autoclavable plasticware/glassware were autoclaved for 30 min. at 121^{o}C for 20 min. (15 lbs/inch2) before use. The culture bottles used in the study were oven dried at 60-80^{o}C temperature before use.

The forceps, scalpels, scissors were sterilized by flaming and were kept in spirit inside laminar air flow chamber for further use.

3.1.4 Stock solutions and their preparations

In the present investigation, *in vitro* studies were carried out on Murashig and Skoog medium (MS medium, 1962). The stock solutions of MS medium salts were prepared as 20 or 200X concentrations in double distilled water. The growth hormones stock solutions were prepared by dissolving in solvents like NaOH or HCl and then final volume was made using distilled water. The stock solutions were then stored in refrigerator at 4ºC for further use. The stock solution of EDTA and $FeSO_4$ was kept in brown colored bottle to protect from photo-oxidation.

Table 3.1: Composition of Murashige and Skoog's (1962) medium

Stock solution (strength)	Stock solution required for one litre of MS medium (ml/l)	Constituent salt	Conc. of salt in one litre of MS medium (mg/l)	Salt required for one litre of stock solution of (g/l)
Stock Solution A (20x)	50 ml	(NH_4NO_3)	1650	33.0
		(KNO_3)	1900	38.0
		($CaCl_2.2H_2O$)	440	8.8
		($MgSO_4.7H_2O$)	370	7.4
		(KH_2PO_4)	170	3.4
Stock Solution B (200x)	5.0 ml	(KI)	0.83	0.166
		(H_3BO_3)	6.2	1.240
		($MnSO_4.4H_2O$)	22.3	4.460
		($ZnSO_4.7H_2O$)	8.6	1.720
		$aMoO_4.2H_2O$)	0.25	0.050
		($CoCl_2.6H_2O$)	0.025	0.005
		($CuSO_4.5H_2O$)	0.025	0.005
Stock Solution C (200x)	5.0 ml	($FeSO_4.7H_2O$)	27.8	5.560
		(Na2-EDTA)	37.3	7.460
Stock Solution D (200X)	5.0 ml	Thiamine HCl	0.1	0.020
		Nicotinic acid	0.5	0.100
		Pyridoxine HCl	0.5	0.100
		Glycine	2.0	0.400
Stock Solution E	5.0 ml	Myo-inositol	100.0	20.00
		Sucrose	30000.0	
		Agar	8000.0	
		pH of medium	5.8	

3.1.5 Media Preparation and sterilization

All the microrpopagation experiments of present investigation were conducted on Murashige and Skoog's medium (1962). The stock solutions of MS medium were mixed in the quantity mentioned in table 3.1 and the final volume was adjusted using

double distilled water. The 1N HCl or 1N NaOH were used to adjust the pH of the medium to 5.8. The agar-agar (8g/l) was dissolved in the medium while boiling with constant stirring. After preparation, medium was poured in the culture bottles and the lids of culture bottles were closed. The culture bottles containing medium were then autoclaved at 121^{o}C temperature and pressure of 15 lbs/inch2 for 15-18 min. After sterilization the medium was stored at room temperature till further use.

3.1.6 Explant Preparation:

The juvenile shoot tips and nodal segments were used as explants. The explants were collected from green house and were washed with liquid detergent followed by washing under running tap water and finally with double distilled water.

3.1.7 Antibacterial and Antifungal management of explants

Excised explants were sterilized with 0.2%-0.4% Streptocyclin (Antibiotic for plant pathogens) for 60-150 min. to remove bacterial contamination and with 0.2%-0.4% solution of bavistin (systemic antifungal agent) for 60-150 min. to get rid of fungal contamination.

3.1.8 Surface sterilization of explants

The explants were surface sterilized in the laminar air flow chamber. The UV light was turned on for 15 min before working in the chamber. After switching off the UV light, the working platform was cleaned with spirit. The pretreated explants were surface sterilized with 0.1% mercuric chloride ($HgCl_2$) with different durations (1-5min.).The explants were then subjected to 4-5 washing with double distilled sterilized water to remove the traces of extra $HgCl_2$.

3.1.9 Inoculation of explants

The surface sterilized explants were then inoculated on to nutrient medium in the culture vessel with the help of sterilized equipment (forceps, scissor etc.). The inoculated explants were then kept at controlled condition in growth chambers at 25±2^{o}C temperature and light intensity of 100 μEM^{-2} sec^{-1} (1000 lux).The 16/8 hours light/ dark period was provided using florescent tubes.

3.2 Growth regulators used for micropropagation studies of *Carica papaya*

MS basal medium fortified with various concentrations and combinations of growth regulators was used in the present investigation for micropropagation study of *Carica papaya*. MS basal medium supplemented with various concentrations of 6-Benzylaminopurine(BAP), Kinetin, 1-Naphthaleneacetic acid(NAA), Indole Butyric Acid(IBA), Indole-3-acetic Acid(IAA), Zeatin, Thiazuron and Giberrilic Acid (GA_3)

were used for establishment (Table 3.2) and for multiplication (Table 3.3). Various additives like silver nitrate, adenine sulphate, polyethylene glycol, putericine and spermidine, were also used (Table 3.4) to study their effect on multiple shoots induction in papaya. The half strength MS medium fortified with different concentration of NAA, IAA and IBA were employed for rooting in elongated shoots of papaya (Table 3.5).

Table 3.2 Composition of MS-medium supplemented with different concentrations of growth regulators used for *in vitro* establishment of papaya variety Pusa Nanha

Media Code						MS+ Hormonal Composition mg/lit		
	BAP (mg/l)	Kinetin (mg/l)	GA_3 (mg/l)	NAA (mg/l)	IBA (mg/l)	Zeatin (mg/l)	TDZ (mg/l)	Medium strength
PE_0	---	---	---	---	---	---	---	Full
PE_1	0.1	---	---	---	---	---	---	Full
PE_2	0.5	---	---	---	---	---	---	Full
PE_3	1.0	---	---	---	---	---	---	Full
PE_4	1.5	---	---	---	---	---	---	Full
PE_5	2.0	---	---	---	---	---	---	Full
PE_6	---	0.1	---	---	---	---	---	Full
PE_7	---	0.5	---	---	---	---	---	Full
PE_8	---	1.0	---	---	---	---	---	Full
PE_9	---	1.5	---	---	---	---	---	Full
PE_{10}	---	2.0	---	---	---	---	---	Full
PE_{11}	---	---	0.5	---	---	---	---	Full
PE_{12}	---	---	1.0	---	---	---	---	Full
PE_{13}	---	---	2.0	---	---	---	---	Full
PE_{14}	---	---	3.0	---	---	---	---	Full
PE_{15}	---	---	4.0	---	---	---	---	Full
PE_{16}	0.5	---	---	0.05	---	---	---	Full
PE_{17}	0.5	---	---	0.1	---	---	---	Full
PE_{18}	0.5	---	---	0.2	---	---	---	Full
PE_{19}	0.5	---	---	0.4	---	---	---	Full
PE_{20}	0.5	---	---	0.8	---	---	---	Full

PE_{21}	0.5	---	---	---	0.05	---	---	Full
PE_{22}	0.5	---	---	---	0.1	---	---	Full
PE_{23}	0.5	---	---	---	0.2	---	---	Full
PE_{24}	0.5	---	---	---	0.4	---	---	Full
PE_{25}	0.5	---	---	---	0.8	---	---	Full
PE_{26}	---	1.0	---	0.05	---	---	---	Full
PE_{27}	---	1.0	---	0.1	---	---	---	Full
PE_{28}	---	1.0	---	0.2	---	---	---	Full
PE_{29}	---	1.0	---	0.4	---	---	---	Full
PE_{30}	---	1.0	---	0.8	---	---	---	Full
PE_{31}	---	---	4.0	0.05	---	---	---	Full
PE_{32}	---	---	4.0	0.1	---	---	---	Full
PE_{33}	---	---	4.0	0.2	---	---	---	Full
PE_{34}	---	---	4.0	0.4	---	---	---	Full
PE_{35}	---	---	4.0	0.8	---	---	---	Full
PE_{36}	---	1.0	---	---	0.05	---	---	Full
PE_{37}	---	1.0	---	---	0.1	---	---	Full
PE_{38}	---	1.0	---	---	0.2	---	---	Full
PE_{39}	---	1.0	---	---	0.4	---	---	Full
PE_{40}	---	1.0	---	---	0.8	---	---	Full
PE_{41}	---	---	---	---	---	0.5	---	Full
PE_{42}	---	---	---	---	---	1.0	---	Full
PE_{43}	---	---	---	---	---	1.5	---	Full
PE_{44}	---	---	---	---	---	2.0	---	Full
PE_{45}	---	---	---	---	---	2.5	---	Full
PE_{46}	---	---	---	---	---	---	0.05	Full
PE_{47}	---	---	---	---	---	---	0.15	Full
PE_{48}	---	---	---	---	---	---	0.45	Full
PE_{49}	---	---	---	---	---	---	1.35	Full
PE_{50}	---	---	---	---	---	---	4.05	Full

Table 3.3 Composition of various media supplemented with different growth hormones used for *in vitro* multiplication of papaya variety Pusa Nanha

Media code	BAP (mg/l)	GA_3	NAA (mg/l)	IBA (mg/l)	IAA (mg/l)	Kinetin (mg/l)	Zeatin (mg/l)	TDZ (mg/l)	Medium strength
PM_0	-	-	-	-	-	-	-	-	Full
PM_1	0.5	1	-	-	-	-	-	-	Full
PM_2	0.5	2	-	-	-	-	-	-	Full
PM_3	0.5	3	-	-	-	-	-	-	Full
PM_4	0.5	4	-	-	-	-	-	-	Full
PM_5	0.5	5	-	-	-	-	-	-	Full
PM_6	0.5	-	0.1	-	-	-	-	-	Full
PM_7	0.5	-	0.2	-	-	-	-	-	Full
PM_8	0.5	-	0.3	-	-	-	-	-	Full
PM_9	0.5	-	0.4	-	-	-	-	-	Full
PM_{10}	0.5	-	0.5	-	-	-	-	-	Full
PM_{11}	0.5	-	-	0.1	-	-	-	-	Full
PM_{12}	0.5	-	-	0.2	-	-	-	-	Full
PM_{13}	0.5	-	-	0.3	-	-	-	-	Full
PM_{14}	0.5	-	-	0.4	-	-	-	-	Full
PM_{15}	0.5	-	-	0.5	-	-	-	-	Full
PM_{16}	0.5	-	-	-	0.1	-	-	-	Full
PM_{17}	0.5	-	-	-	0.2	-	-	-	Full
PM_{18}	0.5	-	-	-	0.3	-	-	-	Full
PM_{19}	0.5	-	-	-	0.4	-	-	-	Full
PM_{20}	0.5	-	-	-	0.5	-	-	-	Full
PM_{21}	0.5	4.0	0.1	-	-	-	-	-	Full
PM_{22}	0.5	4.0	0.2	-	-	-	-	-	Full
PM_{23}	0.5	4.0	0.3	-	-	-	-	-	Full
PM_{24}	0.5	4.0	0.4	-	-	-	-	-	Full
PM_{25}	0.5	4.0	0.5	-	-	-	-	-	Full
PM_{26}	-	1.0	0.2	-	-	1.0	-	-	Full
$PM_{27'}$	-	2.0	0.2	-	-	1.0	-	-	Full
$PM_{28'}$	-	3.0	0.2	-	-	1.0	-	-	Full
$PM_{29'}$	-	4.0	0.2	-	-	1.0	-	-	Full
$PM_{30'}$	-	5.0	0.2	-	-	1.0	-	-	Full
$PM_{31'}$	-	1.0	-	-	-	1.0	-	-	Full
$PM_{32'}$	-	2.0	-	-	-	1.0	-	-	Full
$PM_{33'}$	-	3.0	-	-	-	1.0	-	-	Full
$PM_{34'}$	-	4.0	-	-	-	1.0	-	-	Full
$PM_{35'}$	-	5.0	-	-	-	1.0	-	-	Full
$PM_{36'}$	-	-	0.1	-	-	1.0	-	-	Full
$PM_{37'}$	-	-	0.2	-	-	1.0	-	-	Full

$PM_{38'}$	-	-	0.3	-	-	1.0	-	-	Full
$PM_{39'}$	-	-	0.4	-	-	1.0	-	-	Full
$PM_{40'}$	-	-	0.5	-	-	1.0	-	-	Full
$PM_{41'}$	-	-	0.1	-	-	-	2.0	-	Full
$PM_{42'}$	-	-	0.2	-	-	-	2.0	-	Full
$PM_{43'}$	-	-	0.3	-	-	-	2.0	-	Full
$PM_{44'}$	-	-	0.4	-	-	-	2.0	-	Full
$PM_{45'}$	-	-	0.5	-	-	-	2.0	-	Full
$PM_{46'}$	-	-	0.1	-	-	-	-	1.35	Full
$PM_{47'}$	-	-	0.2	-	-	-	-	1.35	Full
$PM_{48'}$	-	-	0.3	-	-	-	-	1.35	Full
$PM_{49'}$	-	-	0.4	-	-	-	-	1.35	Full
$PM_{50'}$	-	-	0.5	-	-	-	-	1.35	Full

Table 3.4 Composition of various media supplemented with additives used for *in vitro* multiplication of papaya variety Pusa Nanha

Media code	**BAP (mg/l)**	**GA_3 (mg/l)**	**$AgNo_3$ (mg/l)**	**Ads (mg/l)**	**PEG (mg/l)**	**Putrescine (mg/l)**	**Spermidine (mg/l)**
PMA_1	0.5	4.0	5.0				
PMA_2	0.5	4.0	10.0				
PMA_3	0.5	4.0	15.0				
PMA_4	0.5	4.0	20.0				
PMA_5	0.5	4.0	25.0				
PMA_6	0.5	4.0		5.0			
PMA_7	0.5	4.0		10.0			
PMA_8	0.5	4.0		15.0			
PMA_9	0.5	4.0		20.0			
PMA_{10}	0.5	4.0		25.0			
PMA_{11}	0.5	4.0			5.0		
PMA_{12}	0.5	4.0			10.0		
PMA_{13}	0.5	4.0			15.0		
PMA_{14}	0.5	4.0			20.0		
PMA_{15}	0.5	4.0			25.0		
PMA_{16}	0.5	4.0				5.0	
PMA_{17}	0.5	4.0				10.0	
PMA_{18}	0.5	4.0				15.0	
PMA_{19}	0.5	4.0				20.0	
PMA_{20}	0.5	4.0				25.0	
PMA_{21}	0.5	4.0					5.0
PMA_{22}	0.5	4.0					10.0
PMA_{23}	0.5	4.0					15.0
PMA_{24}	0.5	4.0					20.0
PMA_{25}	0.5	4.0					25.0

Table 3.5 Compositions of MS half strength medium supplemented with various types of auxins with varying concentrations used for *in-vitro* rooting

Media code	MS+ Hormonal composition (mg/l)			
	IBA	**NAA**	**IAA**	**Medium strength**
R_0	-	-	-	Half
R_1	1.5	-	-	Half
R_2	2.0	-	-	Half
R_3	2.5	-	-	Half
R_4	3.0	-	-	Half
R_5	3.5	-	-	Half
R_6	-	1.5	-	Half
R_7	-	2.0	-	Half
R_8	-	2.5	-	Half
R_9	-	3.0	-	Half
R_{10}	-	3.5	-	Half
R_{11}	-	-	1.5	Half
R_{12}	-	-	2.0	Half
R_{13}	-	-	2.5	Half
R_{14}	-	-	3.0	Half
R_{15}	-	-	3.5	Half

3.3 Data analysis

3.3.1 Shoot induction experiment

The following data were recorded for percent shoot induction:

$$\text{Percent shoot induction} = \frac{\text{No. of responding explants}}{\text{Total no. of explants cultured}} \times 100$$

3.3.2 Multiplication and Additives experiment

The regenerated shoots were sub-cultured on MS basal medium containing various concentrations of growth regulators and data were recorded for time taken for multiple shoots induction from each culture. The experimental design was completely randomized design (CRD) and all the data recorded were statistically analyzed using SPSS statistical program. Each experiment was carried out in at least nine replications for each treatment. Data were then subjected to analysis of variance (ANOVA) and means were compared using Duncan's Multiple Range Tests ($P \leq 0.05$) with Harmonic Mean Sample Size = 3.0.

3.3.3 Root induction and hardening experiment

Well elongated shoots of Pusa Nanha were aseptically placed on different medium composed of MS basal (half strength) with various concentrations of auxins. Data for percentage root induction were recorded as follows

$$\text{Percent root induction} = \frac{\text{No. of shoots showing roots induction}}{\text{Total no. of shoots cultured}} \times 100$$

Rooted plantlets were transferred in the pots containing different potting mixture. Pots were placed in the green house for hardening.

S. No.	Code of Potting Mixture	Composition
1	PM_1	Sand
2	PM_2	Sand + FYM (1:1)
3	PM_3	Sand + Soil + FYM (1:1:1)
4	PM_4	Sand + Soil + Vermi Compost (1:1:1)

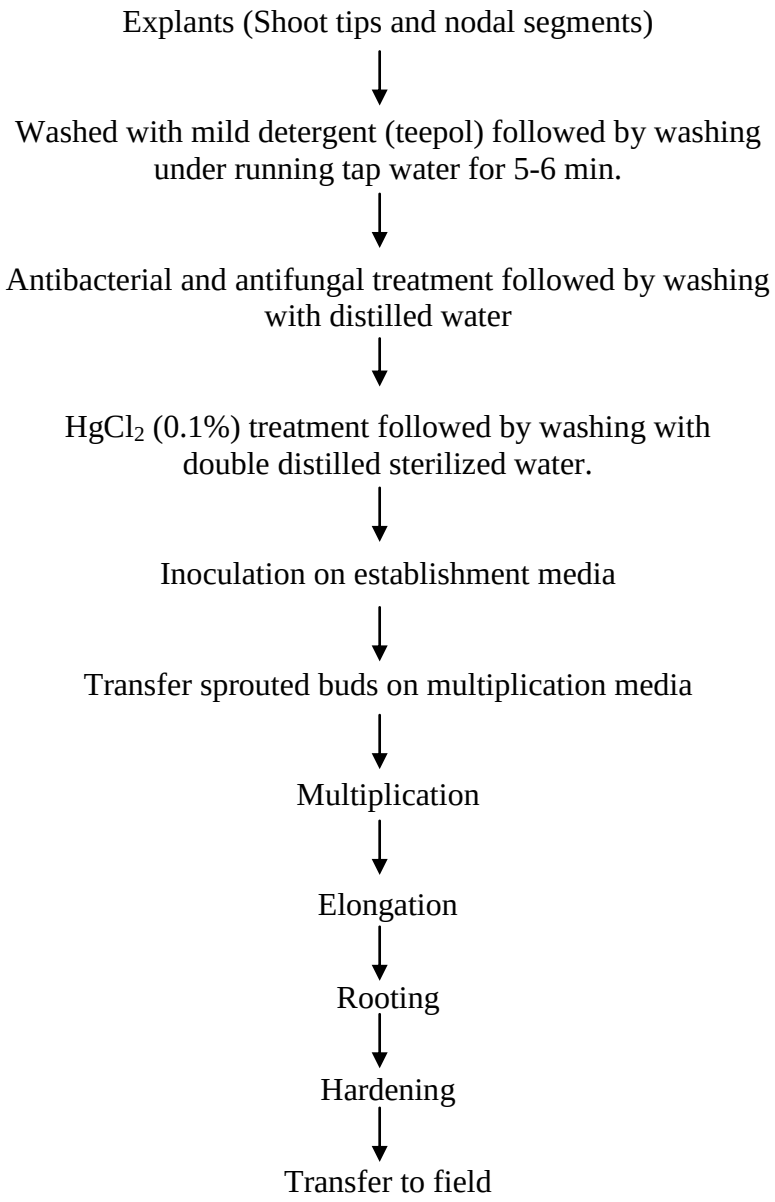
3.3.4 Observations recorded

1. Percent regeneration of explants at different interval of hormonal supplementation in MS medium.

$$\text{Percent regeneration} = \frac{\text{No. of explants showed regeneration}}{\text{Total no. of explants cultured}} \times 100$$

2. Time taken for Nodal explants establishment in Pusa Nanha.
3. Number of buds obtained from each culture in establishment.
4. Number of shoots obtained during multiplication.
5. Percent root induction.
6. Time taken for root initiation.

3.3.5 Flowchart for micropropagation of *Carica papaya* L.



3.4 Genetic fidelity/stability testing of tissue cultured raised plants of *Carica papaya*

3.4.1 Sample

To test the genetic fidelity/stability of tissue cultured raised plants of *Carica papaya*, the DNA was isolated from mother plant and 17 randomly selected tissue culture raised plants of papaya. The genetic fidelity/stability of these tissue cultured raised plants were tested using 15 RAPD and 15 ISSR primers (Table 3.6)

Table 3.6 Different RAPD primers along with their sequence used for testing of genetic fidelity

Sr. No.	Primer	Sequence (5'-3')
1	OPH-3	AGTCGTCCCC
2	OPH-19	CTGACCAGCC
3	RAPD-5	ACCCCCGAAG
4	OPA-01	CAGGCCCTTC
5	OPA-2	TGCCGAGCTG
6	OPA-6	GGTCCCTGAC
7	OPA-9	GGGTAACGCC
8	OPA-13	CAGCACCCAC
9	OPB-07	GGTGACGCAG
10	OPD-03	GTCGCCGTCA
11	OPG-02	GGCACTGAGG
12	OPG-03	GAGCCCTCCA
13	OPG-13	CTCTCCGCCA
14	301	CGGTGGCGAA
15	308	AGCGGCTAGG

Table 3.7 Different ISSR primers along with their sequence used for testing of genetic fidelity

Sr. No.	Primer	Sequence (5'-3')
1	AY1	CTCTCTCTCTCTCTCTGC
2	AY6	CACCACCACGC
3	AY10	CTCTCTCTCTCTCTCTTG
4	AY11	CACACACACACACACAG
5	AY12	TCTCTCTCTCTCTCTCC
6	AY13	ACACACACACACACACG
7	AY21	GAGAGAGAGAGAGAGAYG
8	AY23	CCACTCTCTCTCTCTCTCT
9	AY25	AGAGAGAGAGAGAGAGT
10	AY26	AGAGAGAGAGAGAGAGG
11	AY27	GAGAGAGAGAGAGAGAC
12	AY30	ACACACACACACACACT
13	AY34	CTCCTCCTCCTCCTCCTC
14	AY37	CAGCAGCAGCAGCAG
15	AY45	CACACACACACACACARG

3.4.2 Chemicals

All the chemicals used in the present study were of analytical/ molecular biology grade and obtained from Hi-Media, IDT. The Taq DNA polymerase, dNTPs,

magnesium chloride, PCR buffer (10X) of Bangalore Genei Pvt. Ltd (Merck), India were used in the present investigation. Total 15 RAPD and 15 ISSR primers purchased from Sigma Aldrich Chemical Pvt. Ltd, India (Table 3.6,3.7)were used for detecting genetic stability of tissue cultured raised plants of *carica papaya* . All these primers were synthesized by Sigma Aldrich Chemical Pvt. Ltd, India.

Table 3.8 Reagents used for isolation of DNA from papaya leaves

CTAB extraction buffer		
S. No.	**Reagents**	**Quantity**
1.	Tris (pH 8.0)	1.0 M
2.	EDTA (disodium, pH 8.0)	0.5 M
3.	Sodium chloride	5 M
4.	CTAB	2.0%
5.	β-mercaptoethanol	0.2%
Washing Solution		
1	Ethanol	70 %
2	Sodium acetate	2.0 M
TE buffer		
	Tris(pH 8.0)	10mM
	EDTA (disodium, pH 8.0)	1 mM
RNase		
	Ribonuclease A	10mg/ml

Table 3.9 Reagents used for electrophoresis of Genomic DNA.

10 X TBE buffer		
S. No.	**Reagents**	**Quantity**
1	Tris	108.0g
2	Boric acid	55.0g
3	0.5M EDTA (pH 8.0)	40.0 ml
	Final volume	1000.0 ml
6 X loading dye		
1	Sucrose	4.0g
2	Bromophenol blue	0.025g
3	Xylene cyanol	0.025g
	Final volume	10.0 ml

3.4.3 DNA isolation of mother plant and tissue cultured raised plants of papaya

Optimization of DNA Extraction protocol:

1) The young leaves of papaya were kept in mortar and pestle in -80°C freezer for two hours. Then the leaf samples were crushed in pre-chilled mortar and pestle.
2) The sample was then transferred into a eppendorf tube with the preheated extraction buffer (Table 3.8).
3) The content was mixed and stirred gently with β Mercaptoethanol.
4) The mixture was incubated in water bath at 65°C temperature and was cooled down followed by adding equal volume of chilled chloroform: isoamyl alcohol (24:1).
5) The eppendorf tube was centrifuged and aqueous phase was mixed with ice cold isopropanol.
6) The DNA pellet was obtained and air dried overnight
7) Finally the DNA pellet was dissolved in 50-100 µl of 1xTE buffer.

8) The optical density of isolated DNA was measured by spectrophotometer. The purity of DNA was calculated by the A260/280 absorbance ratios obtained. The genomic DNA was visualized by electrophoresis using 0.8% agarose gel (Table 3.9). The gels were photographed using a Gel Documentation system (BIORAD).

3.4.4 PCR Amplifications (ISSR and RAPD):

The isolated DNA of *Carica papaya* was subjected to Polymerase chain reaction (PCR). PCR was performed with 25 µl reaction volume. The reagent concentration was standardize for PCR, which includes template DNA, deoxynucleotide triphosphate, primers, $MgCl_2$, taq buffer and Taq DNA polymerase (Table 3.10). The amplification cycle consist following steps, denaturation step at 94^0 C, followed by 40 cycles of denaturation at 94^0 C, annealing and extension at 72^0 C. For ISSR and RAPD primers, annealing temperature were standardized and amplification cycle was run as shown in Table 3.12. in an Eppendorf thermal cycler. The PCR products were then run on 1.5% agarose gel at 70V for 2h for complete separation of bands. The gel was scanned through Gel Doc System.

TABLE3.10: PCR Reagents for testing of genetic stability (RAPD)

SR.NO.	CHEMICAL	CONCENTERATION
1	dNTPs	140,160 µM
2	$MgCl_2$	1.5,2.0 mM
3	Taq buffer	1X
4	TaqDNA polymerase	1,1.5 unit
5	Primer (RAPD)	5,10,12 µM

TABLE 3.11: PCR Reagents for testing of genetic stability (ISSR)

SR.NO.	CHEMICAL	CONCENTERATION
1	dNTPs	140,160 µM
2	$MgCl_2$	1.5,2.0 mM
3	Taq buffer	1X
4	TaqDNA polymerase	1,1.5 unit
5	Primer(ISSR)	5,10,12 µM

TABLE 3.12: PCR amplification cycle for RAPD primer

Sr.no.	PCR cycle	Temperature	Time (minutes)
1	Initial denaturation	94^0 C	**4**
2	Denaturation	94^0 C	**1**
3	Annealing	45^0 C	**1**
4	Extension	72^0 C	**1**
5	Final Extension	72^0 C	**7**

TABLE 3.13: PCR amplification cycle for ISSR primer

Sr. No.	PCR cycle	Temperature	Time (minutes)
1	Initial denaturation	94^0 C	**2**
2	Denaturation	94^0 C	**1**
3	Annealing	50^0 C	**1**
4	Extension	72^0 C	**4**
5	Final Extension	72^0 C	**7**

The RAPD and ISSR were scored visually, based on presence (taken as 1) or absence (taken as 0) of bands.

3.5 GENETIC DIVERSITY

3.5.1 Collection of varieties

Thirteen (13) papaya varieties and five unknown genotypes were collected from various regions (Table 3.14) of India to assess their genetic diversity using RAPD and ISSR markers.

Table 3.14 Papaya known varieties and unknown genotypes collected from various regions of India

Sr. no.	Varieties	Source
1	Pusa Nanha	IARI, New Delhi
2	Tripura Selection 1	IARI, New Delhi
3	Pune Selection 3	IARI, New Delhi
4	Pant Papaya	GBPAUT, Pantnagar
5	Co7	CISH, Lucknow
6	Pusa Dwarf	SVBPU, Meerut
7	Washington	SVBPU, Meerut
8	Pusa Giant	SVBPU, Meerut
9	Pusa Delicious	SVBPU, Meerut
10	Pusa Majesty	SVBPU, Meerut
11	PS1	SVBPU, Meerut
12	Strain1	SVBPU, Meerut
13	Strain2	SVBPU, Meerut
14	Strain3	SVBPU, Meerut
15	Strain 4	Hisar
16	Strain 5	Sirsa
17	CO2	SVBPU, Meerut
18	PANT2	SVBPU, Meerut

3.5.2 Chemicals

A total of 20 RAPD and 20 ISSR primers were used to study genetic polymorphism in papaya. All these primers were synthesized by Sigma Aldrich Chemical Pvt. Ltd, India.

Table 3.15 ISSR primers used for studying genetic diversity in papaya collected from different regions of India

S.No.	Primer	Sequence (5'-3')
1.	AY1	CTCTCTCTCTCTCTCTGC
2.	AY6	CACCACCACGC
3.	AY10	CTCTCTCTCTCTCTCTTG
4.	AY11	CACACACACACACACAG
5.	AY12	TCTCTCTCTCTCTCTCC
6.	AY13	ACACACACACACACACG
7.	AY21	GAGAGAGAGAGAGAGAYG
8.	AY23	CCACTCTCTCTCTCTCTCT
9.	AY25	AGAGAGAGAGAGAGAGT
10.	AY26	AGAGAGAGAGAGAGAGG
11.	AY27	GAGAGAGAGAGAGAGAC
12.	AY28	CACACACACACACACAG
13.	AY29	GTGTGTGTGTGTGTGTC
14.	AY30	ACACACACACACACACT
15.	AY31	AGAGAGAGAGAGAGAGYT
16.	AY32	GAGAGAGAGAGAGAGAYC
17.	AY34	CTCCTCCTCCTCCTCCTC
18.	AY37	CAGCAGCAGCAGCAG
19.	AY45	CACACACACACACACARG
20.	AY22	CACACACACACACACAVT

Table 3.16 RAPD primers used for studying genetic diversity in papaya collected from different regions of India

S.No.	Primer	Sequence (5'-3')
1.	OPH 3	AGTCGTCCCC
2.	OPH 19	CTGACCAGCC
3.	RAPD 5	ACCCCCGAAG
4.	OPA 1	CAGGCCCTTC
5.	OPA 2	TGCCGAGCTG
6.	OPA 3	AGTCAGCCAC
7.	OPA 6	GGTCCCTGAC
8.	OPA 9	GGGTAACGCC
9.	OPA 10	GTGATCGCAG
10.	OPA 13	CAGCACCCAC
11.	OPA 16	AGCCAGCGAA
12.	OPB 7	GGTGACGCAG
13.	OPB 15	GGAGGGTGTT
14.	OPD 3	GTCGCCGTCA
15.	OPG 2	GGCACTGAGG
16.	OPG 3	GAGCCCTCCA
17.	OPG 13	CTCTCCGCCA
18.	301	CGGTGGCGAA
19.	308	AGCGGCTAGG
20.	310	GAGCCAGAAG

3.5.3 Optimization DNA extraction protocol and PCR Amplifications (ISSR and RAPD):

For genetic diversity, similar protocol was optimized as in genetic fidelity testing (3.4.3). Also, PCR amplification cycle for RAPD (table 3.12) and ISSR (table3.13) was used optimized as earlier for genetic stability experiments.

3.5.4 Data analysis

Based on RAPD and ISSR data, genetic similarity was evaluated by ‘Jaccard’ similarity coefficient by the primers for all genotypes. The cluster tree analysis was

done to estimate relationship among genotypes.

The data generated from the polymorphic fragments were analyzed by Nei and Li (1979) equation

$$\text{Similarity (F)} = \frac{2Mx}{My + Mz}$$

Dissimilarity = 1-F

Where,

Mx = Number of shared fragments between genotypes y and z.

My = Number of scored fragments of genotype y.

Mz = Number of scored fragments of genotypes z.

The genetic distance was calculated using 'Simqual' sub-programme of NTSYS-PC software (numerical taxonomy and multivariate analysis system programme)

Dendrogram was constructed using distance matrix by the unweighed pair-group method with arithmetic average (UPGMA) sub-programme of NTSYS-PC. Diagrams in 2-dimensions and 3- dimensions were constructed.

3.6 Sex determination in different genotypes of *Carica papaya*.

3.6.1 Collection of varieties

The Five varieties of papaya vizPusaNanha , Pant Papaya ,Pant2 ,PS1 and Pusa dwarf were collected from different regions of India (Table 3.17).All the above varieties were dioecious and were maintained in the greenhouse of CPB,Hisar.

TABLE 3.17 Varieties of papaya used for sex determination

Sr. no.	Varieties	Source
1	PusaNanha	IARI.New Delhi
2	Pant Papaya	GBPAUT,Pantnagar
3	PANT2	SVBPU,Meerut
4	Pusa Dwarf	SVBPU,Meerut
5	PS1	SVBPU,Meerut

Table 3.18: SCAR and ISSR markers sequence used for sex determination

Sr. no.	Marker	Sequence	Source
1	SCAR1 (C09)	FP –CTCACCGTCCATTTTAATTA	Niroshini *et al.* (2008)
		RP-CTCACCGTCCGCGGCATCAATGTA	
2	SCAR2 (T12)	FP-GGGTGTGTAGGCACTCTCCTT	Deputy *et al.* (2002)
		RP-GGGTGTGTAGCATGCATGATA	
3	SCAR3 (W11)	FP-CTGATGCGTGTGTGGCTCTA	Deputy *et al.* (2002)
		RP-CTGATGCGTGATCATCTACT	
4	ISSR	$(GACA)_4$	Gangopadhyay *et al.* (2007)

3.6.2 Reaction conditions for ISSR Primers

The PCR was run for sex determination using SCAR and ISSR markers shown in table 3.18 with their sequences and source. The amplification cycle was optimized for them shown in Table 3.19 and 3.20.

Table 3.19: PCR amplification cycle for ISSR primer

Sr.no.	PCR cycle	Temperature	Time (minutes)
1	Initial denaturation	94^0 C	**2**
2	Denaturation	94^0 C	**1**
3	Annealing	50^0 C	**1**
4	Extension	72^0 C	**4**
5	Final Extension	72^0 C	**7**

TABLE 3.20: PCR amplification cycle for SCAR primer

Sr. No.	PCR cycle	Temperature	Time (minutes)
1	Initial denaturation	94^0 C	**5**
2	Denaturation	94^0 C	**1**
3	Annealing	58^0 C	**1**
4	Extension	72^0 C	**1**
5	Final Extension	72^0 C	**7**