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RESEARCH ARTICLE

Micropropogation of Dioceious *Carica papaya* L. varieties and Analysis of genetic stability using Random Amplified Polymorphic DNA (RAPD) Markers

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ABSTRACT

A micropropagation protocol was developed for four dioecious varieties (Honey dew, CO-2. Madhu and Taiwan) of Carica papaya L. using axillary meristem explants from field grown plants. Explants were cultured on various induction and proliferation media for multiple shoot production and on elongation media for shoot elongation. IBA dip treatments of cut shoot ends produced healthy roots. A combination of soil: vermiculite: cowdung (1:1:1) was used as a substrate for hardening of plantlets. Genetic stability of micropropagated clones was evaluated using Random Amplified Polymorphic DNA (RAPD) analysis. Both random primers (AM 765819 and AM 75004) and sex specific primers (OPF2 male specific and Npf76 & Npf77 female specific) primers were used for the analyses. A total of seven samples were analyzed taking one mother plant varieties and six plantlets derived from it in each variety. The random primers produced monomorphic banding patterns in all the varieties and the micropropagated plantlets obtained from them, thus, proving their genetic similarity. The same banding pattern of the mother and micropropagated plantlets using sex specific markers showed similarity at the sexual level and thus, demonstrated that micrpropagation resulted in production of the plantlets of same sex as that of mother plant. The age of the plantlets had no effect on the RAPD results.

Key Words: *Tissue culture, papaya, axillary meristem, proliferation, RAPD*

INTRODUCTION

A majority of papaya plantations are established from seed using dioecious cultivars which, means they are out bred. Due to out breeding, plants are not true to type and exhibit significant variation in yield, fruit quality and disease susceptibility within cultivated populations (Drew, 1988). Additionally, as sex cannot be determined until the mid-development stage, several seedlings are planted in each position and eventually thinned, retaining only the most vigorous female plants with one male to every 10 to 20 female as pollinators. A need to replant every three years to ensure quality fruit production (Samson, 1986), propagation by seed represents a significant cost to the producer.

Micropropagation is the only economic method of constantly producing new uniform planting material of known sex. Several micropropagation techniques were developed (Yie and Liaw, 1977; Litz and Conover, 1978), but none proved to be commercially acceptable. Drew and Smith (1986) developed a nodal culture technique, which has found some commercial acceptance. However, premature leaf senescence during culture remains a major practical limitation preventing this technique from becoming commercially adopted. *In vitro* regeneration of papaya through organogenesis and somatic embryogenesis has been achieved using a variety of explants (Rajeevan and Pandey, 1986; Winner, 1988; Reuveni *et al*. 1990; Hossain *et al.* 1993; Chen *et a*l., 1987; Fitch, 1993; Bhattacharya *et al.,* 2002).

Due to occurrence of somaclonal variations during micropropagation, homogeneity and identity of tissue cultured plants with stock plants cannot be certified. (Corniquel and Mercier, 1994). Assessment of genetic stability of *in vitro* derived plants is essential step for production of true to type clones (Diaz *et al.,* 2003). Identification of off types and variants at an early stage of development necessitates use of molecular markers for this purpose (Taylor

et al., 1995). RAPD markers have proved to be useful for the detection of genetic changes (Soliman, 2003; Taylor *et al.*, 1995; Anand, 2003).

In view of the above, present study was undertaken keeping mainly the following objectives in mind:

1. To investigate the possibility of obtaining plantlets of same sex as that of the mother plant employing tissue culture techniques.
2. To develop a protocol for rapid multiplication of plantlets of known sex.

MATERIAL AND METHODS

1. PLANT MATERIAL

Healthy field grown plants of CO-2, Honey Dew, Madhu and Taiwan papaya varieties were selected after flowering. Axillary meristem explants from male and female plants were maintained separately. Meristems were washed thoroughly with water three times for 5 min followed by soap solution for 15 min and 3 times with water for 5 min. Explants were surface sterilized with 1% mercuric chloride solution inside a laminar flow cabinet followed by 3 washes with sterile distilled water. Explants were treated with GA_3 (500 mg/l) for 1 h and then transferred to MS medium containing 400 mg/l magnesium sulphate for establishment. The pH of the media was adjusted to 5.8 prior to addition of 0.75% agar and autoclaved. Cultures were placed at 25 ± 2^0C under cool white fluorescent light at 25µMm^{-2}s^{-1} with a 16h and 8 h photoperiod.

2. INDUCTION OF SHOOTS

The meristems which remained green and showed some swelling and after 15 days were used for shoot bud induction were inoculated on various media such as-

1. MS basal medium;
2. MS medium fortified with auxin (NAA and IAA) individually (0.1, 0.5, 1.0, 2.0, 3.0, 4.0 and 5.0 mg/l);
3. MS medium with AS (adenine sulphate) (1.0, 2.0, 3.0, 4.0 and 5.0 mg/l);
4. MS medium fortified with higher range of AS (10, 20, 40, 60, 80 and 100 mg/l);
5. MS fortified with various concentrations of auxins or cytokinins in different combinations BAP + NAA; AS + NAA; BAP + NAA + AS;
6. Combination of cytokinins: BAP + KIN; BAP + AS; KIN + AS and BAP + KIN + AS and combination of auxins NAA + IAA.
7. Also MS medium fortified with two cytokinins and one auxin was also used. The cultures were incubated under conditions mentioned above.

Twenty explants were treated in each experiment.

3. SHOOT MULTIPLICATION

Axillary meristems which responded to induction were then inoculated on various media for proliferation of shoots buds. The concentrations of BAP, NAA and AS were standardized based on the result of induction medium. The cultures were incubated under conditions mentioned above.

4. SHOOT ELONGATION

Multiple shoot buds of same length (1.0 cm) were later separated and elongated on various elongation media such as MS and half strength MS were used. Also MS media with various growth regulators GA_3, IAA and NAA (0.1, 0.5, 1.0, 2.0, 3.0, 4.0 and 5.0 mg/l) were used. Also multiplication media MS + BAP (5.0 mg/l) + AS (10 mg/l) + NAA (2mg/l) + Sucrose 6% supplemented with coconut milk (CM- 5, 10, 15 and 20%); casein hydrolysate (CH- 50 and 100 mg/l) and urea (0.1 and 0.5 mg/l) were also used for elongation.

The standardized elongation media was as follows:

EM = MS + BAP (5.0 mg/l) + AS (10 mg/l) + NAA (2mg/l) + CM (10%) + Sucrose 6%

5. ROOTING

Shoots (4-5 cm in length) with leaves (6-7 in number) were inoculated on MS medium fortified with various concentrations of NAA, IAA and IBA (1.0, 2.0, 3.0, 4.0 and 5.0 mg/l) individually or the base of the shoot dipped in various concentrations of NAA IAA or IBA (250, 500, 1500, 2000, 2500 and 3000mg/l) for 10, 20 or 30 seconds and then inoculated on MS medium for rooting. The cultures were incubated for three weeks.

6. HARDENING AND ACCLIMATIZATION

Rooted plantlets were hardened in pots containing soil vermiculite and cow dung (1:1:1) at 25 ± 2^{0}C under diffused light (16h light period and eight h) for four weeks in a net house and gradually transferred to the field.

7. RAPD ANALYSES

DNA was isolated from leaves of the source and micropropagated plants as described before (Doyle and Doyle, 1987) with minor modifications (Padamlatha and Prasad, 2006). DNA yield was measured using a UV spectrophotometer (ND -1000 at 260 nm). DNA purity was estimated by calculating the absorbance ratio at 260: 280 nm. DNA concentration and quality was also determined by running samples on 0.8% agarose gel and band intensities were compared with lambda DNA marker using earlier methods (Sambrook *et al.*, 1989). Isolated DNA samples were diluted to a concentration of 1µg/µl.**A** PCR mix was made by mixing 12.50µl red dye, 1.0µl primer, 11µl sterile distilled water and 0.5µl template DNA to a final volume of 25.0µl. The mix was vortexed and centrifuged briefly. The negative control consisted of the reaction mix without template DNA (negative control) and a positive control without enzyme. Amplification was carried out out by PCR as described earlier (Williams *et al.*, 1990). It was as follows:

Table 1: PCR Program

I	II			III			IV
94˚C	94˚C	55˚C	72˚C	94˚C	58˚C	72˚C	72˚C
5 min	45sec	1 min	1.5min	45sec	1min	1min	10min
Denaturation	8 cycle			35 cycle			Final Extension

Once the PCR was complete, loaded PCR products were analyzed by running them on a1% agarose gel at 50-100V (5-6 V/cm gel) for 1:30h. Following electrophoresis, gels were analyzed at 302nm wavelength on a gel documentation system. Images were taken and analyzed using Alpha View® Software.

RESULTS

IN VITRO PROPAGATION OF PAPAYA FROM AXILLARY MERISTEM

INITIATION

Initiation of multiple shoot bud in all the four varieties (CO-2, Honey Dew, Madhu and Taiwan) did not take place when axillary meristem explants were inoculated on MS basal medium and MS basal medium fortified individually or with singly or various concentrations of IAA and/or NAA (0.1-5.0 mg/l); BAP and/or KIN (0.1-5.0 mg/l) and AS in two ranges (1.0-5.0 mg/l and 10.0-100.0 mg/l). MS medium fortified with auxins (0.1-5.0 mg/l) and cytokinins (0.1-3.0 mg/l) in various combinations resulted in excessive callus formation.

MS medium fortified with BAP, AS and IAA; KIN, AS and NAA or KIN, AS and IAA resulted in the initiation of the shoot buds with excessive development of callus at the base of the axillary meristem explant.

The MS medium fortified with (4-5 mg/l) BAP, (1-2 mg/l) NAA and AS (10-20 mg/l) acted as initiation medium.

In the initiation medium, AS (0.5-5mg/l) did not result in the initiation of multiple shoots and AS (20-100 mg/l) resulted in stress symptoms. In all the varieties of *Carica papaya* L the

axillary meristem explants inoculated on the initiation medium fortified with BAP (5.0 mg/l), AS (10mg/l) and NAA (2mg/l) gave rise shoot buds (Table 2, Fig. 1-13)

Table 2: Number of shoot buds per axillary meristem explant on initiation media in different varieties of papaya

Medium	Conc. of BAP (mg/l)	Conc. of AS (mg/l)	Conc. of NAA (mg/l)	Varieties			
				No. of shoot buds			
				CO-2	Madhu	Honey dew	Taiwan
MS medium with 0.75% agar pH 5.8	4	10	2	2.00±0.25	1.50±0.17	1.25±0.15	2.75[a]±0.29
	4	20	2	1.00±0.25	0.75±0.15	0.50±0.17	1.25±0.15
	5	10	2	2.50±0.17	1.75±0.29	1.50±0.17	3.00[b]±0.25
	5	20	2	0.75±0.15	0.50±0.17	0.25±0.15	1.00±0.25

[a-b] Significantly different at 5% level of probability.

Fig. 1: Response of axillary bud meristem of different varieties of papaya innoculated on different media for initiation of shoot buds

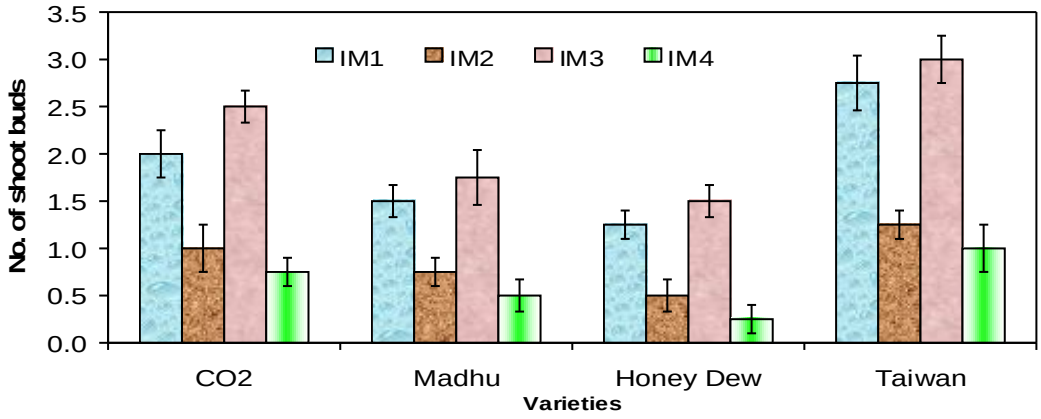


Fig. 2: Established axillary meristem of var. Taiwan after treatment with autoclaved GA_3 after 15 days of culture on MS medium.

Fig. 3: Bud breaking from axillary meristem of var. Taiwan after treatment with autoclaved GA3 after 30 days of culture on MS medium

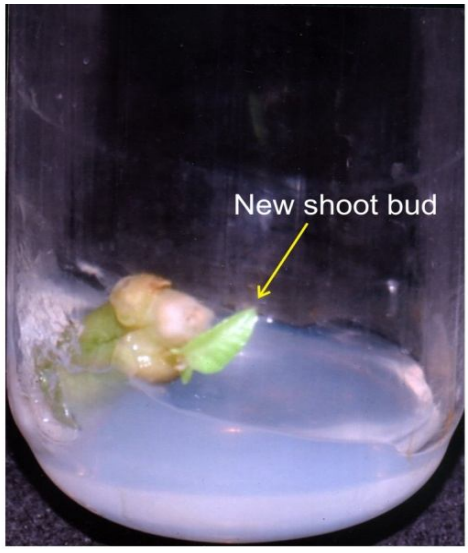


Fig. 4: Established axillary meristem of var. Honey Dew after treatment with autoclaved GA_3 after 15 days of culture on MS medium.

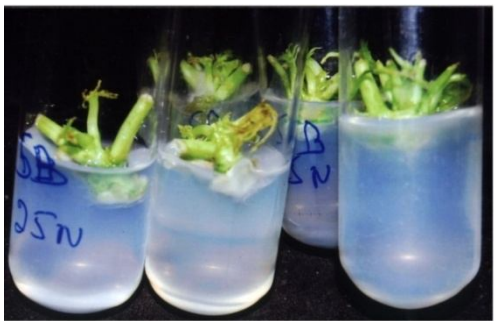


Fig. 5: Established axillary meristem of var. CO-2 after treatment with autoclaved GA3 after 15 days of culture on MS medium

Fig. 6: Induction of shoot formation (var. Taiwan) when inoculated on IM3 medium after 15 days of culture

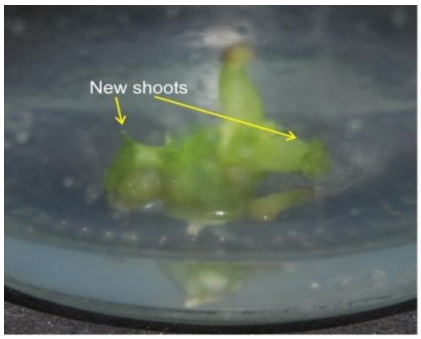


Fig. 7: Induction of shoot formation (var. Madhu) when inoculated on IM3 medium after 15 days of culture

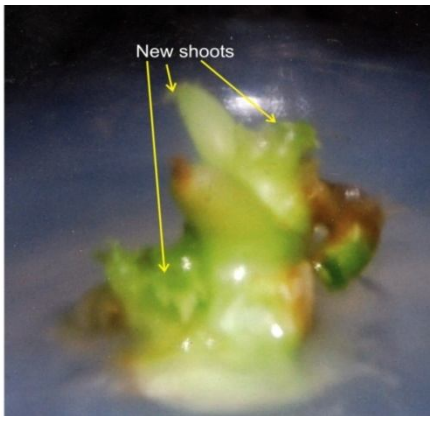


Fig. 8: Induction of shoot formation (var. CO-2) on IM3 medium after 15 days of culture

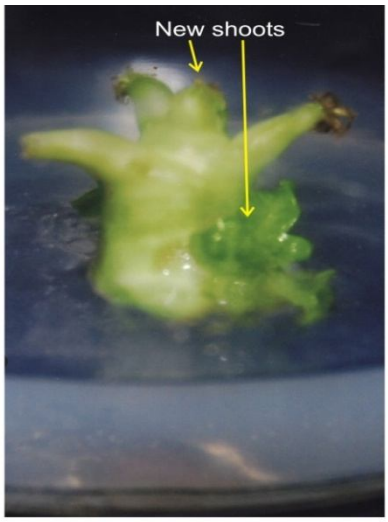


Fig. 9: Induction of shoot formation (var. CO-2) on IM3 medium after 30 days of culture

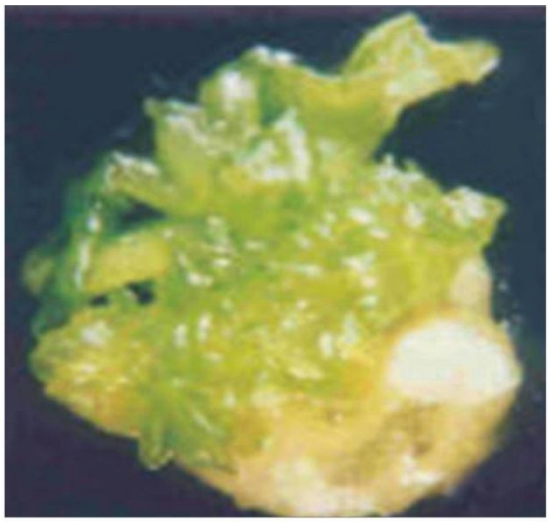


Fig.10: Induction of shoot formation in axillary meristem (var. Madhu) after 15 days of culture in MS medium containing BAP (5mg/l)+AS (10mg/l)+NAA (2mg/l).

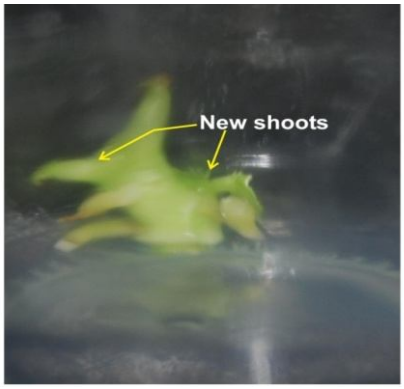


Fig. 11: Induction of shoot formation in axillary meristem (var. Honey Dew) after 15 days of culture in MS medium containing BAP (5mg/l)+AS (10mg/l)+NAA (2mg/l).

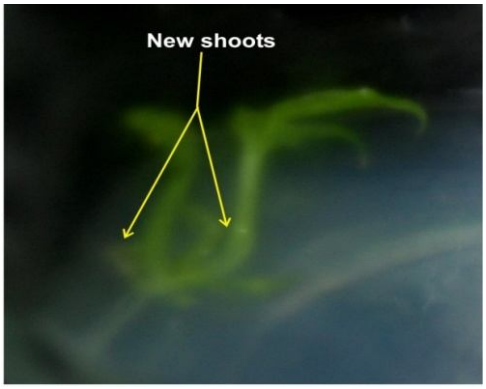


Fig. 12: Induction of shoot formation in axillary meristem (var. Taiwan) after 15 days of culture in MS medium containing BAP (5mg/l)+AS (10mg/l)+NAA (2mg/l).

Fig. 13: Proliferation of shoot formation in axillary meristem (var. Taiwan) after 15 days of culture in MS medium containing BAP (5mg/l)+AS (10mg/l)+NAA (2mg/l)+sucrose (6%).

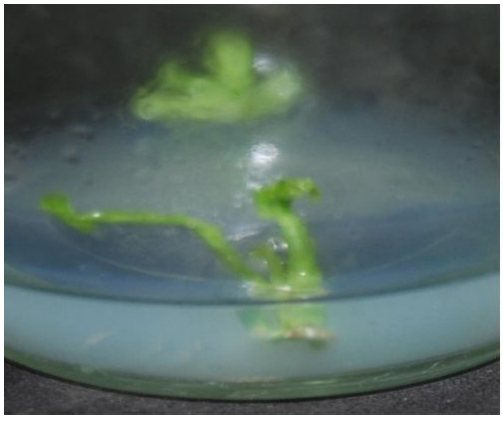


Fig. 14: Proliferation of shoots from axillary meristem (var. Taiwan) after 30 davs of culture in PM3.

Fig. 15: Proliferation of shoots from axillary meristem (var. Madhu) after 45 days of culture in PM3

MULTIPLICATION OR PROLIFERATION OF SHOOTS

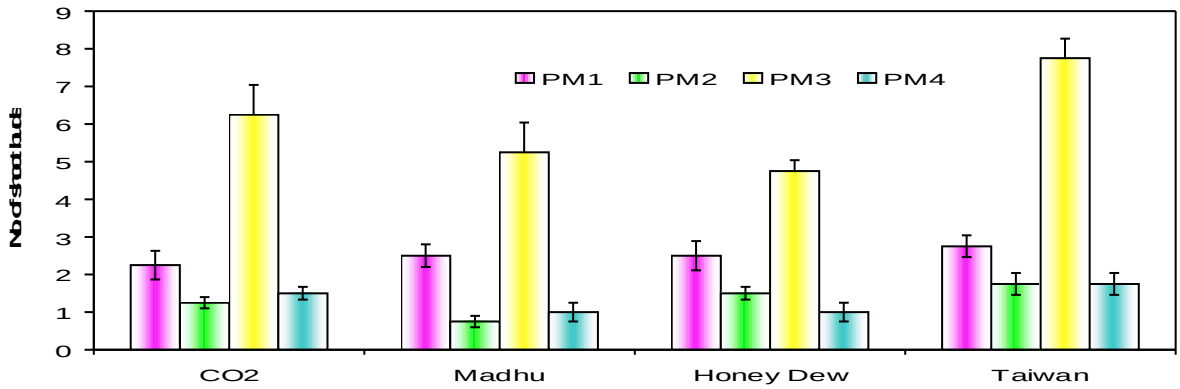
The axillary meristem explants which showed shoot induction were inoculated on the proliferation medium i.e MS medium fortified with 6% sucrose, BAP (5.0 mg/l), AS (10 mg/l) and NAA (2mg/l). These meristems later developed into shoots after 5-6 weeks of incubation. Among the various combinations used in the above multiplication media PM3 medium i.e MS + BAP (5.0 mg/l) + AS (10 mg/l) + NAA (2mg/l) + 6% sucrose was found to be most effective. No response was observed in explants cultured on control medium. In all the cases, shoots arose directly from the explant without any intermediary callus growth. The number of shoots developed from each explant was found to be dependent on concentration of sucrose and growth regulators. The results are presented in Table 3, Fig. 14-16.

Table 3: Number of shoot buds per axillary meristem explant on proliferation media in different varieties of papaya

Medium	Conc. of BAP (mg/l)	Conc. of AS (mg/l)	Conc. of NAA (mg/l)	Varieties			
				CO-2	Madhu	Honey dew	Taiwan
				No. of shoot buds			
MS medium with 6% sucrose 0.75% agar pH 5.8	4	10	2	2.25 ± 0.38	2.50 ± 0.30	$2.50^{(a)}$ ± 0.39	2.75 ± 0.29
	4	20	2	1.25 ± 0.15	0.75 ± 0.15	1.50 ± 0.17	1.75 ± 0.29
	5	10	2	$6.25^{(a)}$ ± 0.79	$5.25^{(a)}$ ± 0.79	$4.75^{(b)}$ ± 0.29	$7.75^{(a)}$ ± 0.52
	5	20	2	1.50 ± 0.17	1.00 ± 0.25	1.00 ± 0.25	1.75 ± 0.29

[a-b] Significantly different at 5% level of probability.

Fig. 16: Effect of proliferation on media on axillary meristem of papaya varieties



SHOOT ELONGATION

The shoot buds failed to elongate when inoculated on MS, MBG and half strength MS media. Proliferation medium supplemented with CM (5%) or sucrose (0-3% and 7-10%) induced excessive callusing at the base of the shoot buds. Similar response was observed when MS medium fortified with GA_3, IAA or NAA and proliferation medium supplemented with CM (15%) was used. Proliferation medium supplemented with casein hydrolysate and urea promoted elongation however; it also resulted in fragile yellowing and shedding of leaves. Thus they were not used for further study. However, proliferation medium supplemented with CM (10%) promoted elongation. The results are presented in Table 4, Fig. 17-19.

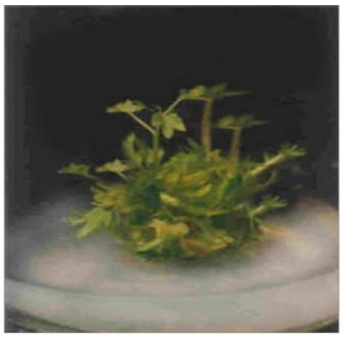
Table 4: Shoot length of different varieties of papaya after eight weeks of culturing on elongation media

Variety	Length in cm
CO-2	$3.00^{(b)}$ ± 0.35
Madhu	$2.50^{(a)}$ ± 0.17
Honey Dew	2.00 ± 0.25
Taiwan	$4.25^{(c)}$ ± 0.45

[a-c] Significantly different at 5% level of probability.

Fig. 17: Elongation of shoots (var. Madhu) when inoculated in MS medium BAP (5mg/l)+ AS (10mg/l)+ NAA (2mg/l)+ CM (10%) +sucrose (6%)

Fig. 18: Elongation of shoots (var. Taiwan) when inoculated in MS medium BAP (5mg/l)+ AS (10mg/l)+ NAA (2mg/l)+ CM (10%) +sucrose (6%).



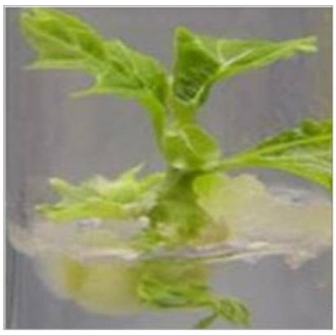
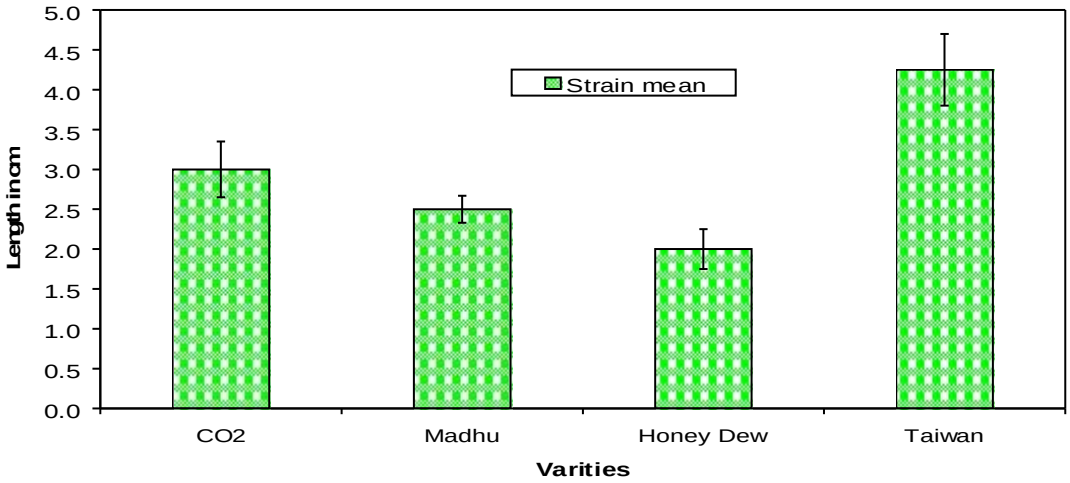


Fig. 19: Effect of elongation media on Axillary meristem of Papaya varieties



ROOTING

Rooting was not observed in shoots inoculated on MS medium fortified with NAA (1-5 mg/l) or the base of the shoot dipped in NAA, IAA or IBA (250-3000 mg/l) for 10 and 20 seconds and inoculated on MS medium.

Profuse callusing was observed at the base of shoots which were dipped in IAA and NAA (250-3000 mg/l) for 30 seconds and inoculated on MS medium.

The shoots inoculated on MS medium fortified with IAA or IBA (1.0-5.0 mg/l) produced roots. However percentage rooting was low and time taken for rooting was more. These rooting media were therefore, not used for further study.

The base of the shoot dipped in IBA (2500 mg/l) for 30 seconds inoculated on MS medium exhibited rooting in all the varieties (Table 5, Fig. 20-25)

Table 5: Effect of IBA (2500mg/l) treatment on rooting of shoots of various varieties of papaya

Variety	Rooting %	Days taken for rooting
CO-2	$91.5^{(c)} \pm 0.39$	$13.5^{(b)} \pm 0.39$
Madhu	$86.5^{(b)} \pm 0.72$	$16.5^{(d)} \pm 0.39$
Honey Dew	$64.5^{(a)} \pm 0.63$	$15.5^{(c)} \pm 0.39$
Taiwan	$94^{(d)} \pm 0.65$	$13.25^{(a)} \pm 0.29$

$^{a-d}$ Significantly different at 5% level of probability

Fig. 20: Rooting percentage in different varieties of *Carica papaya* L.

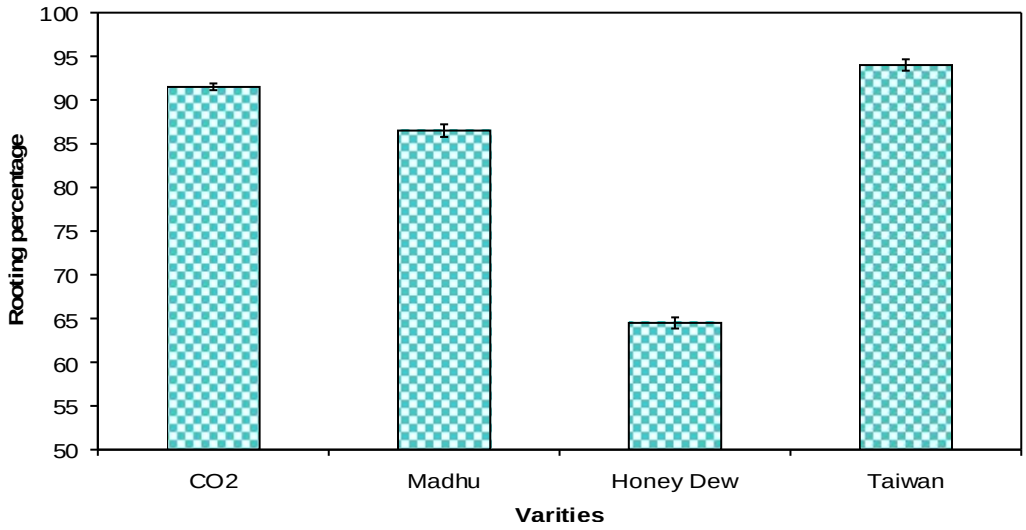


Fig.21. No. of days taken for rooting by different varieties of papaya

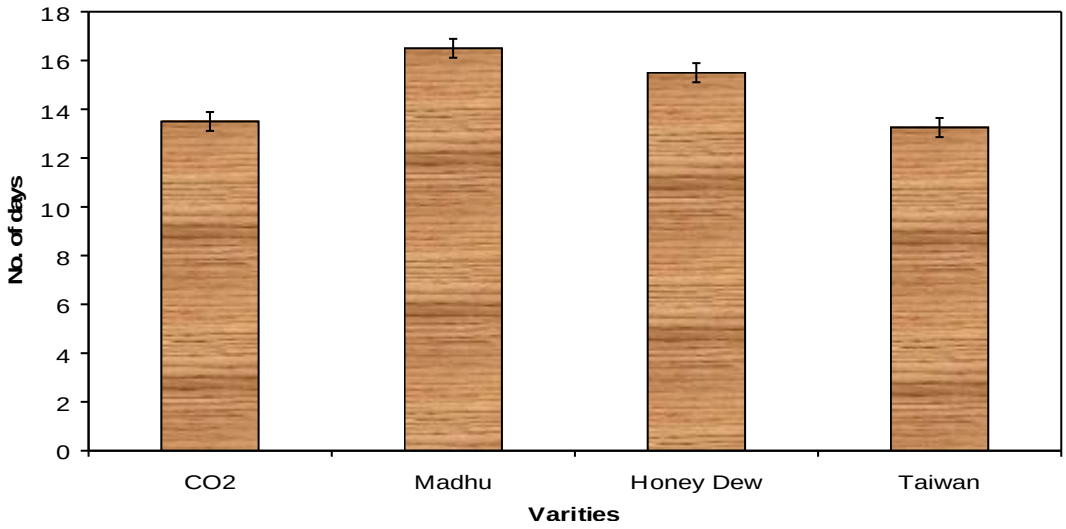


Fig. 22: 45 days old rooted culture of var. Madhu (rooting obtained by dipping the shoots in 2500 mg/l of IBA and then inoculating on MS medium).

Fig. 23: 45 days old rooted culture of var. Honey Dew (rooting obtained by dipping the shoots in 2500 mg/l of IBA and then inoculating on MS medium).

Fig. 24: Normal rooting of plantlet when it was dipped in 2500 mg/l of IBA and then inoculating on MS medium (var. Taiwan)

HARDENING

Mixture of soil, vermiculite and cow dung (1:1:1 w/w) used as substratum showed survival of plantlets in different varieties of papaya in four weeks (Table 5, Fig. 25 and 26)

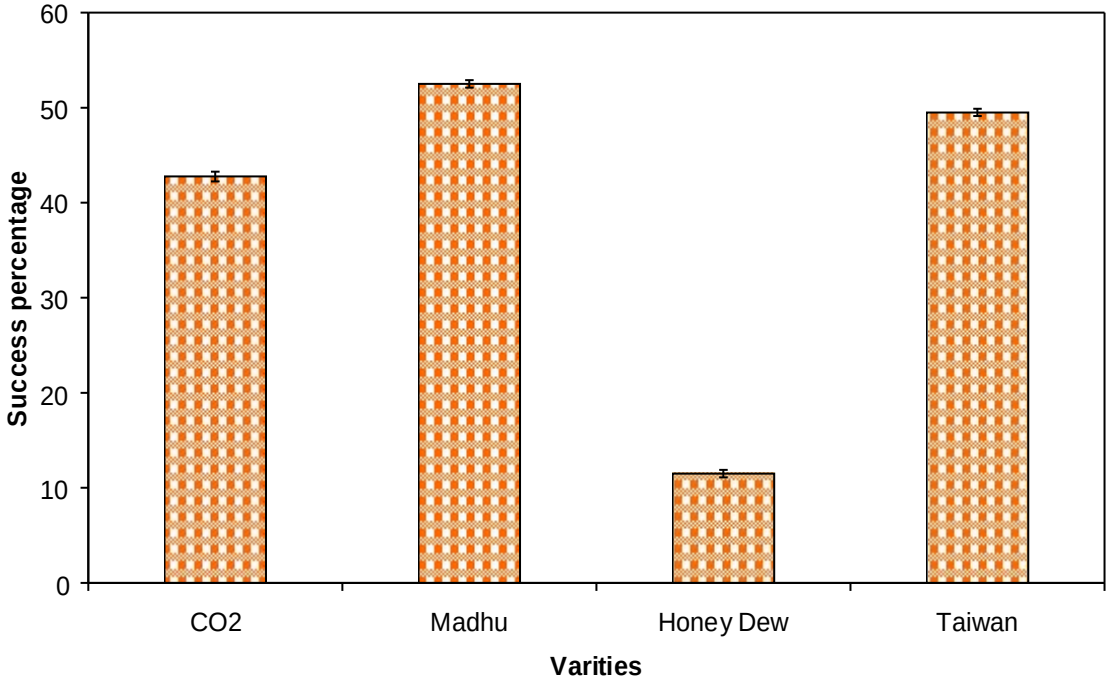
Table 6: Hardening percentage in plantlets of various varieties of papaya

Variety	Hardening %age
CO-2	42.75$^{(b)}$±0.52
Madhu	52.50$^{(d)}$±0.39
Honey Dew	11.50$^{(a)}$±0.39
Taiwan	49.50$^{(c)}$±0.39

$^{a-d}$ Significantly different at 5% level of probability.

Fig. 25: Hardening of tissue culture Plantlets in 8cm pots

Fig.26. Hardening success percentage in different varieties of papaya



RAPD ANALYSIS OF THE FOUR VARIETIES OF PAPAYA

In order to confirm the genetic integrity of micropropagated papaya plantlets belonging to different varieties, the leaves were obtained from the healthy field grown plants belonging to different varieties maintained over a period of one year. Then they were subjected to RAPD analysis using two primers AM 765819 (Fig. 27-30) and AM 750044 (Fig 31-34). A total of seven samples were analyzed taking one mother plant varieties and six plantlets derived from it in each variety.

Fig. 27: PCR banding pattern in mother plant and plantlets obtained from it using primer AM 765819 in variety Honey Dew.

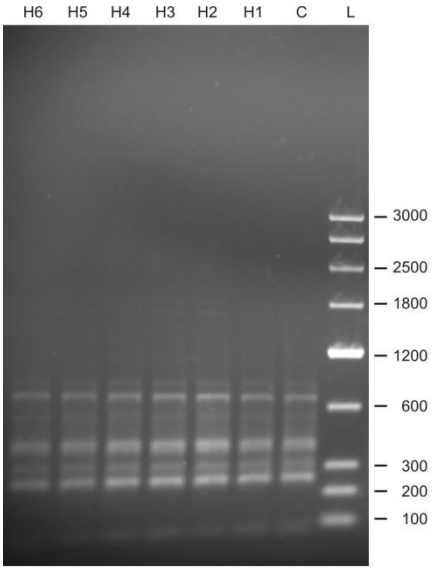


Fig. 28: PCR banding pattern in mother plant and plantlets obtained from it using primer AM 765819 in variety CO-2.

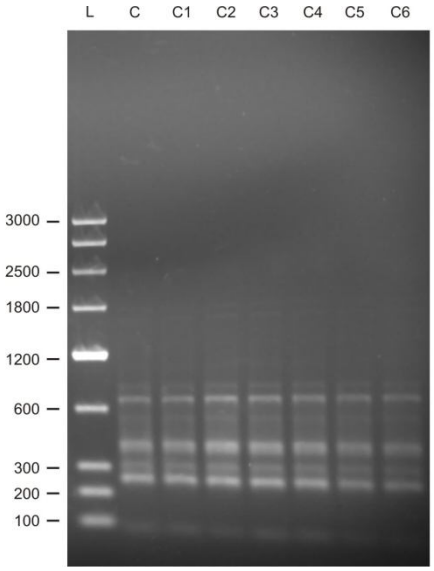


Fig. 29: PCR banding pattern in mother plant and plantlets obtained from it using primer AM 765819 in variety Madhu.

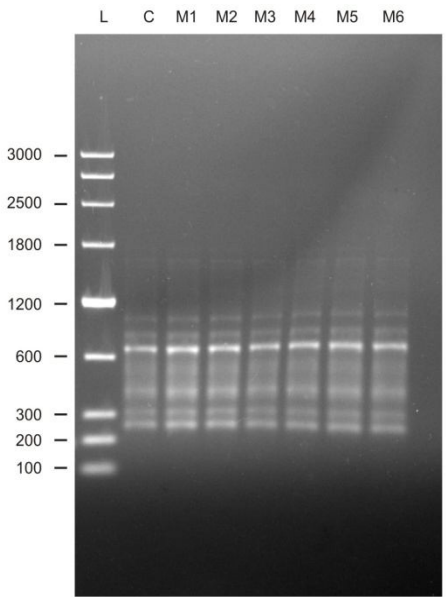


Fig. 30: PCR banding pattern in mother plant and plantlets obtained from it using primer AM 765819 in variety Taiwan.

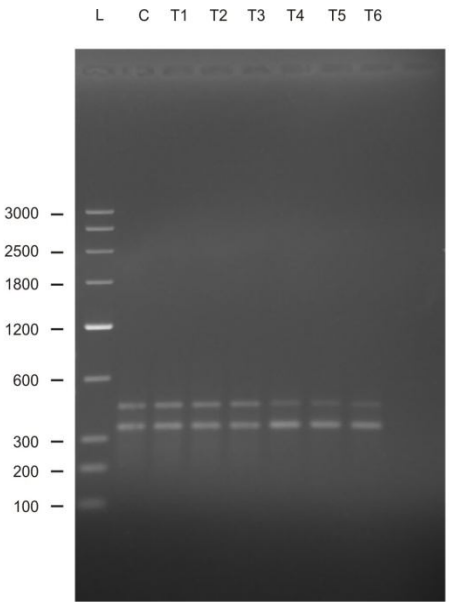


Fig. 31: PCR banding pattern in mother plant and plantlets obtained from it using primer AM 750044 in variety Honey Dew.

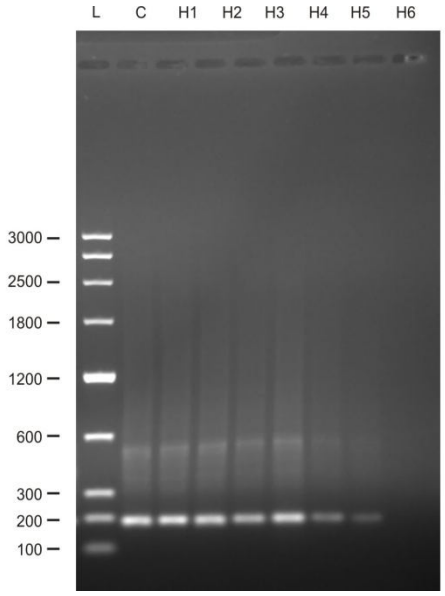


Fig. 32: PCR banding pattern in mother plant and plantlets obtained from it using primer AM 750044 in variety Madhu.

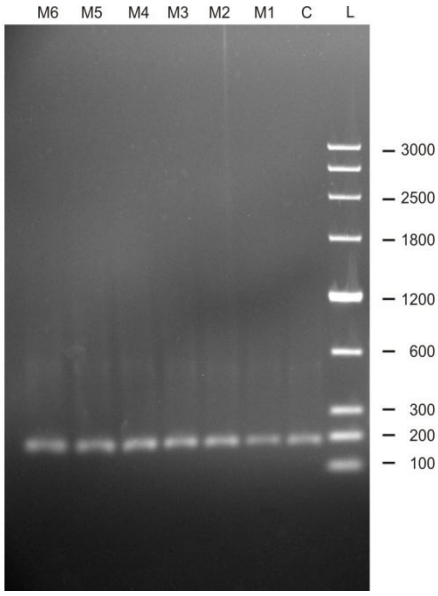


Fig. 33: PCR banding pattern in mother plant and plantlets obtained from it using primer AM 750044 in variety CO-2.

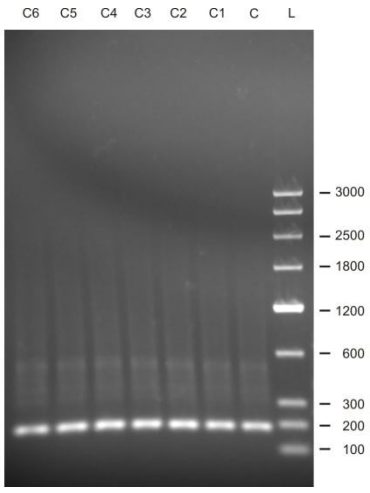
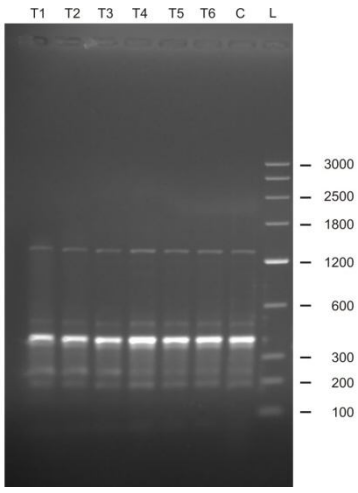


Fig. 34: PCR banding pattern in mother plant and plantlets obtained from it using primer AM 750044 in variety Taiwan.



The RAPD analysis showed that the mother plant and the micropropagated plantlets shared similar banding patterns. No RAPD polymorphism was observed in the micropropagated plantlets.

The primer AM 765819 produced three bands near 250bp, 400bp and 700bp in three varieties i.e. Honey Dew, Madhu and CO-2 and the plantlets raised from them. The variety Taiwan produced two bands between 300bp and 600bp region. The primer AM 750044 produced a band near 200bp in three varieties i.e Honey Dew, Madhu and CO-2 and the plantlets obtained from them. The variety Taiwan showed two bands one near 400bp region and another near 1300bp region.The ladder used in every case was from 100bp to 3000bp.

The data generated by RAPD analysis using two primers i.e AM 765819 and 750044 was also used to prepare UPGMA dendrograms. It was observed that the two groups are formed. The first group consisted of only one variety i.e Taiwan and the second group consisted of three varieties Madhu, CO-2 and Honey Dew (Fig. 35 and Fig. 36).

Fig. 35: UPGMA dendogram generated by primer AM 765819 in different varieties of papaya

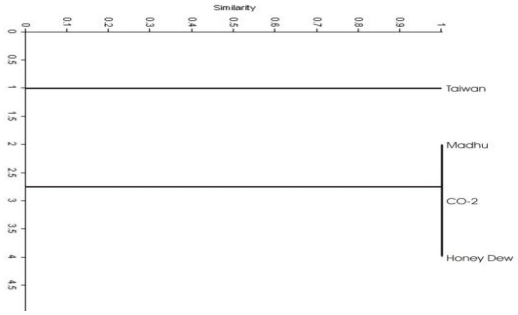
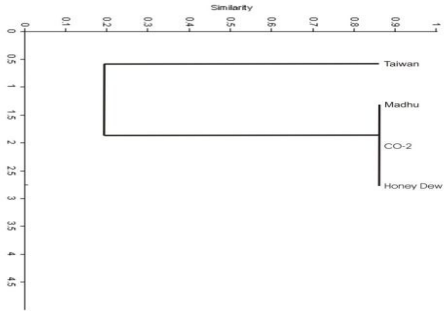


Fig. 36: UPGMA dendogram generated by primer AM 750044 in different varieties of papaya



RAPD ANALYSIS FOR SEX DETERMINATION IN PAPAYA

Three sex specific primers namely OPF2, Napf 76 and Napf 77 (Parasnis *et al.*, 2000) were used for determining sex of the mother plant (i.e. the control) and the micropropagated plantlets obtained from it. OPF2 was male specific primer and produced a single band if the papaya plant in question is a male. Napf 76 and Napf 77 when used together produced two bands provided the papaya plant in question was a female. DNA isolated from leaves of the mother plant and at least six to eight of micropropagated plantlets obtained from it was subjected to PCR amplification using the three primers. In Honey Dew the mother plant and all the six of the plantlets showed amplification with Napf76 and 77 but none of them responded to OPF2 (Fig. 37)indicating that they are all female. In Madhu the mother plant and all the six of the plantlets showed amplification with Napf 76 and 77 producing two bands (Fig. 38) but none of them responded to OPF2 indicating that they all were female. The same was true for Taiwan (Fig. 39). In CO-2 however, all the plantlets including the mother plant produced a single band with OPF2 (Fig. 40) but none of them responded to Napf 76 and 77. This showed that all the plantlets and the mother plant were male.

Fig. 37: DNA amplification of the mother plant and micropropogated plantlets using NPf 76 and NPf 77 in variety Honey Dew of papaya (M= mother plant, H_1-H_6 plantlets)

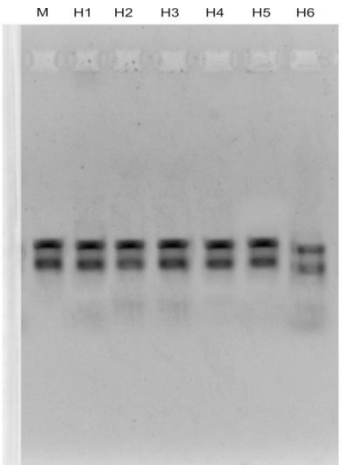


Fig. 38: DNA amplification of the mother plant and micropropogated plantlets using NPf 76 and NPf 77 in variety Madhu of papaya (M= mother plant, M_1-M_6 plantlets)

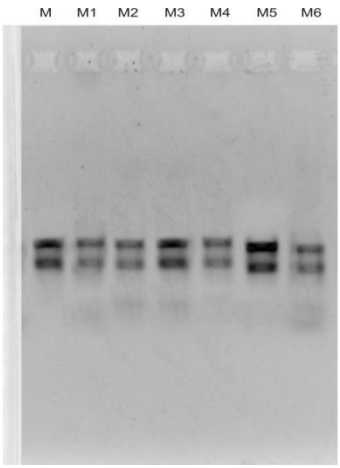


Fig. 39: DNA amplification of the mother plant and micropropogated plantlets using NPf 76 and NPf 77 in variety Taiwan of papaya (M= mother plant, T_1-T_6 plantlets)

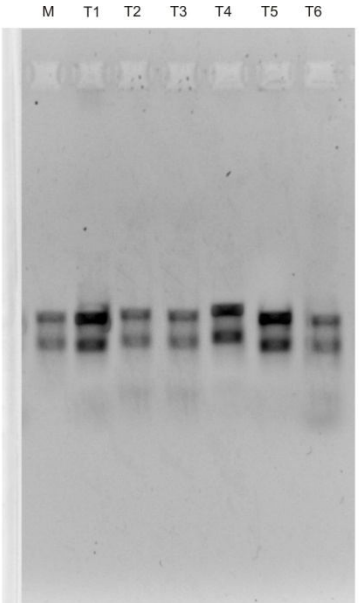
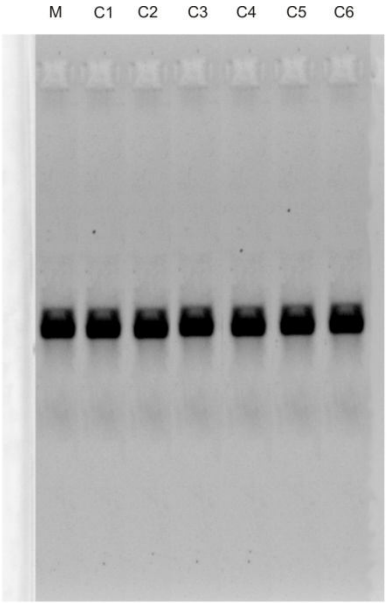


Fig. 40: DNA amplification of the mother plant and micropropogated plantlets using OPF-2 in variety CO-2 of papaya (M= mother plant, C_1-C_6 plantlets)



DISCUSSION

In vitro propagation

In vitro propagation in papaya was achieved via organogenesis using axillary meristems as the explants.

Organogenesis using axillary meristem as explant

The axillary meristems of four varieties viz Honey Dew, Madhu, CO-2 and Taiwan were treated with (500 mg/l) GA_3 for one hour and then inoculated onto MS medium with $MgSO_4$ (400 mg/l) for establishment. GA_3 is known to enhance growth in plants (Razdan, 2007). Among the various media tested for induction of shoots MS medium fortified with BAP (4-5 mg/l), NAA (1-2 mg/l) and AS (10-20 mg/l) acted as induction medium. The IM3 medium (MS + BAP 5.0 mg/l + AS 10 mg/l + NAA 2mg/l) acted as the best induction medium. In case of axillary meristem explants of papaya adenine sulphate was found necessary for induction and multiplication of shoot buds (Saha *et al.,* 2004). Adenine sulphate acts as a cytokinin and organic nutrient source (Razdan, 2007).

The axillary meristem explants which showed shoot induction were inoculated onto induction medium supplied with 6% sucrose. The induction medium containing higher amount of sucrose 6% (as compared to normal 3%) acted as the proliferation media. The axillary meristem explants of four varieties Honey Dew, Madhu, CO-2 and Taiwan produced maximum number of shoots in PM3 media i.e. MS + BAP (5.0 mg/l) + AS (10 mg/l) + NAA (2mg/l) + Sucrose 6%. The carbon source in culture medium has been reported to influence proliferation (Enakasha *et al.,* 1993) and morphogenesis of cultures (Eapen and George, 1993). Sucrose has been the most commonly used carbohydrate in papaya tissue culture studies (Rajeevan and Pandey, 1986; Mondal *et al.* 1990). Positive effect of sucrose on shoot proliferation was reported earlier (Sul and Korban, 1998).

Elongation of shoots obtained from axillary meristem explants

The shoots obtained from axillary meristem explants either failed to elongate or produced excessive callusing in various media tested for elongation. Proliferation medium

supplemented with CH (Caesin hydrolysate) and urea produced some elongation but it was accompanied with fragile yellowing and shedding of leaves. The best elongation medium was found to be MS medium containing BAP (5mg/l), NAA (2mg/l), AS (10mg/l) and coconut milk (10%). The use of CM (Coconut Milk) in elongation medium for papaya shoots is also reported by Saha *et al.* (2004). CM (Coconut Milk) has been reported to act as an organic source as well as a cytokinin supplement in culture media (Razdan, 2007).

Rooting in shoots obtained from axillary meristem

Profuse callusing was observed at the base of shoots which were dipped in IAA and NAA (250-300 mg/l) for 30 seconds and inoculated onto MS medium. The shoots inoculated on MS medium fortified with IAA or IBA (1.0-5.0 mg/l) showed low rooting percentage and time taken for rooting was also more. The base of the shoots dipped in IBA (2500 mg/l) for 30 seconds inoculated onto MS medium exhibited rooting in all varieties. The auxins (IBA and NAA) have been used in papaya shoots to induce roots *in vitro* (Drew, 1987; Drew *et al.*, 1993). IBA was superior in terms of rooting percentage and number of roots per shoot when the basal medium was supplemented with riboflavin (Drew, 1987; Drew *et al.*, 1993). In papaya rooting has usually been achieved using agar solidified medium *in vitro* (Drew, 1988; Teo and Chan, 1994).

RAPD ANALYSIS IN PAPAYA

In our study RAPD analysis of micropropagated plants of *Carica papaya* L showed a profile similar to that of the control indicating that no genetic variation had occurred *in vitro*. RAPD based assessment of genetic stability of *in vitro* grown micropropagated plants have been reported in many other plant species (Rout *et al.*, 1998; Rout and Das, 2002; Martins *et al.*, 2004; Venkatachalam *et al.*, 2007). Use of axillary meristems as explants for micropropagation lowers the risk of genetic instability. The results are in agreement with those of Shenoy and Vasil (1992) who reported that the micropropagation through explants containing organized meristem is generally associated with low risk of genetic instability as they are more resistant to genetic changes under *in vitro* conditions.

In our study of papaya the length of the culture period (over one year) with regular sub culturing did not affect their genetic integrity. Similar results were also reported by Martins *et al.*, (2004) in almonds plantlet and Angel *et al.*, (1996) in cassava plantlets. Some authors, however, have reported that the time in *in vitro* culture could promote somaclonal variation (Orton, 1985; Hartman *et al.*, 1989; Nayak and Sen, 1991). Veddrame *et al.*, (1999) concluded that, genetic variation in a culture line could be affected more by the genotype than by the period in culture. Hammerschlag *et al.*, (1987) suggested that the genotype and the nature of explants could influence the phenotype stability of the plants obtained, in studies performed with micropropagated peach plantlets. Culture time does not seem to be the only parameter affecting genetic stability (Gould, 1986)

SEX DETERMINATION IN PAPAYA

Sondur *et al*., (1996) constructed a genetic linkage map for *Carica papaya* L. based on random amplified polymorphic DNA (RAPD) markers. They constructed a genetic linkage map of papaya using 596 RAPD reaction primers. In doing so they discovered a locus which determines sex in papaya trees. They suggested that the OPT12 and OPT iC markers flank a 14cM region corresponding to the SEX 1 locus which is inherited as a dominant form.

Lemos *et al*., (2002) used RAPD analysis to differentiate between the sexual forms of three commercial *Carica papaya* cultivars belonging to the Solo group. RAPD assays using the BC 210 primer (TCTCGGTG) were able to detect hermaphrodites in all the cultivars tested. They also suggested that the BC 210_{438} molecular marker (CACCGAGG) was much better at papaya sex determination at seedling stage before they are planted in the field.

Urasaki *et al.*, (2002) used the RAPD technique to determine the sex of *Carica papaya* L. with three sex types male, female and hermaphrodite. They constructed a SCAR marker which

was specific to males and hermaphrodites. They also proved that SCAR is a suitable marker for the precise and rapid diagnosis of sex in papaya.

Parasnis *et al.*, (2000) reported a PCR based seedling sex diagnostic assay (SSDA) designed specially for early sexing of papaya seedlings. They developed a male specific SCAR marker in papaya by cloning a male specific RAPD (831 bp) fragment and designed longer primers.

Deputy *et al.*, (2002) developed molecular marker tightly linked to SEX 1, the gene that determines plant sex in papaya *Carica papaya*. They cloned three RAPD products and sequenced a portion of their DNA. Based on these sequences three SCAR primers were synthesized. SCAR T12 and SCAR W11 produced products in hermaphrodite and male plants and rarely in females. SCAR I produced products in all papaya plants regardless of the sex. Hence, a PCR based system for rapid and reliable sex determination in papaya plant from small tissue samples was developed.

Bedoya and Nuñez (2007) used RAPD markers to determine the sex of Colombian cultivars of dioecious papaya genotypes. It was found that a RAPD marker of 900 bp is present in male plant which is absent in female or hermaphrodites. From this RAPD marker a sequence characterized amplified region (SCAR) was developed and it was possible to amplify fragments from the genome of male and hermaphrodites and not the females ones.

In our study OPF2 male specific and Napf 76 and Napf 77 female specific primers were used. The mother plant and the plantlets obtained from it in the variety CO-2 showed amplification using OPF2 primer indicating that they were all males. Rest of the varieties and their micropropagated plantlets showed amplification with Napf 76 and Napf 77 producing two bands. Thus proving they were all females. The study also proved that tissue culture has no affect on the sex of tissue culture raised plant and they are true to the type.

This method is very useful for obtaining true to type plants i.e. males from male and females from female, thus, exhibiting the possibility of sex determination at early stage in papaya and making the whole process cost effective. This should however, tested for its reproducibility before exploiting it at commercial level.

The shoot buds developed during micropropagation from axillary meristem can serve as substitute to somatic embryos in preparing synthetic seeds. The shoot buds can be excised during the multiplication stage and coated with suitable coating material. The synthetic seed so obtained can be made to develop into plantlet using suitable media.

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