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Tissue Culture Protocol for In-vitro Propagation of Papaya (*Carica papaya* L.)

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ABSTRACT

Papaya is slowly emerging from the status of a homestead crop to that of commercial crop due to increasing awareness of multifold uses of papaya. Its cultivation is encountered with the problem of its dioecious nature. Micro propagation represents the only economic way of continuously producing uniform planting material of known sex. The studies on *in- vitro* propagation of papaya (*Carica papaya* L.) was carried out at the Department of Pomology and Floriculture, College of Agriculture, Vellayani. The propagation studies were carried out by enhanced release of axillary buds in Papaya variety Pusa Nanha. Apical buds and lateral buds from seedlings and mature plants were used as explant. Explants were subjected to different treatments of plant growth substances for culture establishment and shoot proliferation. The study revealed that full strength MS medium supplemented with sucrose 30.0 g/l and agar 6.5 g/l under light condition produced highest shoot number and longest shoot in papaya. Application of BA 0.50 mg/l along with NAA 0.1mg/l was found to be better for initial culture establishment and proliferation. Application of amino acid, glycine, 100.00 mg/l resulted in highest shoot proliferation rate, while highest shoot length was obtained from arginine100.0 mg/l. Addition of activated charcoal 0.05 per cent and Cobalt chloride 5.0 mg/l increased the shoot proliferation rate and shoot length in papaya. *In vitro* rooting was more in full strength MS medium supplemented with IBA 3.0 mg/l, sucrose 30.0 g/l and activated charcoal 0.05 per cent.

Key Words : Explant, Micropropagation,Medium, Papaya, Shoot proliferation.

INTRODUCTION

Papaya (*Carica papaya* L.) is one of the major tropical fruit crops suited for both nutrition gardens and commercial orchards. Nutritive value, high yielding potential, year round fruiting behaviour and short pre-bearing period make papaya unique among fruit crops. Due to its multifold uses it is slowly emerging from the status of a homestead crop to that of commercial crop.

Papaya cultivation is encountered with the problem of its dioecious nature. The majority of plantations are established from seeds using dioecious cultivars. Hence seed propagation results in seedlings which are either male or female. However, the setback of propagating by seeds is the production of non-true-to-type planting materials due to the segregation of off springs at the second filial generation, the inherent heterozygosity and dioecious nature of the plant, and the seeds of open-pollinated flowers exhibit considerable variation in shape, size and flavour and susceptibility to diseases. Moreover, as sex cannot be determined until the mid development stage, three seedlings are established in each planting position, till flowering. Then they are thinned, retaining only the most vigorous female plant with one male to every 10 to 20 female plants. This results in wastage of inputs. With a requirement to renew plantations every three year to ensure quality fruit production propagation by seed represents a significant cost to the producer.

The multiplication rates are low in the vegetative propagation methods like mound layering. Similarly asexual reproductions are also often tedious and impractical when carried out on

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large scale. Therefore to minimize these problems, efficient micro propagation of papaya has become critical for the multiplication of specific sex types of papaya. This field level problem necessitated the substitution of seedling progeny with tissue culture propagules developed from female or bisexual plants. Micro propagation represents the only economic way of continuously producing uniform planting materials of known sex (Chan and Teo, 2002) However, suitable protocols for tissue culture of papaya are to be developed to suit specific situations. Hence the present experiment was conducted with an objective of refining the existing tissue culture protocol of papaya.

MATERIALS AND METHODS

The studies on *in vitro* propagation of papaya (*Carica papaya* L.) were carried out at the Department of Pomology and Floriculture, College of Agriculture, Vellayani using papaya variety Pusa Nanha. Apical buds and lateral buds from seedlings and mature plants were used as explant for *in vitro* propagation. The explants were collected from mature plants and one month old seedlings. For surface sterilization apical buds were treated with mercuric chloride (0.08 %) for 10 min. and lateral buds for 12 min. with intermittent shaking.

Explants from papaya were subjected to different treatments of plant growth substances for culture establishment. In order to standardize a suitable hormone combination for better culture establishment, studies were carried out using BA, Kinetin and NAA at various concentrations. The treatments involved were different levels of cytokinins, *viz*; BA (0.2 -5.0mg/l) and Kinetin (0.5-5.0mg/l) alone or in combination with auxin, *viz.,* NAA (0.01, 0.1 and 0.5 mg/l). Six replications were kept for each treatment. The basal media used for the study were MS and half strength MS. Observations were recorded on the number of surviving cultures (percentage), number of cultures showing initial growth, number of days for bud initiation and number of buds per culture, after four weeks of culturing in the establishment media. Plant growth substances like BA (0.2-5.0 mg/l) and Kinetin (0.5-5.0 mg/l) alone or in combination with NAA (0.01, 0.1 and 0.5 mg/l) were tried for shoot proliferation. Six replications were also kept for each treatment. The number of cultures survived (percentage), number of shoots per culture and length of the longest shoot and abnormalities in shoot growth if any, were recorded after six weeks of culturing in the shoot proliferation media.

The cultures were kept in light or in darkness in order to assess the effect of light on multiple shoot proliferation. Trials on the *in vitro* rooting were conducted in full MS medium. Individual shoots measuring 2.50-3.50 cm length, excised from shoot proliferating cultures were subjected to different rooting treatments with varying levels of IBA (0.5 - 4.0 mg/l), IAA (0.1- 2.0 mg/l) and NAA (0.1 – 3.0 mg/l). Each treatment was replicated six times. Observations on the number of cultures initiating roots, number of days for root initiation, number of roots, root length and abnormality in root growth, if any, were recorded four weeks after culturing.

RESULTS AND DISCUSSION

Various workers reported micro-propagation in papaya by enhanced release of axillary buds (Lai *et al*, 2000; Chan and Teo, 2002 and Hidaka *et al,* 2008). Explants used in the present study were apical buds and lateral buds of mature plants and one month old seedlings. Panjaitan *et al* (2007) also used similar method by using shoot tips from seedlings and lateral buds from female plants for *in vitro* propagation of papaya. Anandan *et al* (2011) also reported that establishment of seedling explants were highest when apical tips of four week old seedlings of papaya were used as explants.

Forty treatments were tried to assess the effect of plant growth substances on culture establishment of papaya. Significant difference was noticed among these treatments. Among the different plant growth substances used in the establishment medium, the earliest bud break was obtained with the application of Kinetin 1.0 mg/l in combination with NAA

Table 1. Effect of plant growth substances on culture establishment medium of papaya.

Treatment	Plant growth substances (mg l-1)	Initial growth (%)	Survival (%)	Number of days taken for bud initiation	Number of buds initiated per culture
CE1	BA 0.00 + NAA 0.01	66.67	50.00	8.50	1.00
CE2	BA 0.00 + NAA 0.10	100.00	66.67	10.33	1.00
CE3	BA 0.00 + NAA 0.50	33.33	33.33	13.33	1.00
CE4	BA 0.20	33.33	33.33	11.00	1.00
CE5	BA 0.20 + NAA 0.01	50.00	50.00	9.17	1.17
CE6	BA 0.20 + NAA 0.10	83.33	50.00	7.33	1.00
CE7	BA 0.20 + NAA 0.50	83.33	83.33	14.83	1.17
CE8	BA 0.50	50.00	33.33	10.17	1.00
CE9	BA 0.50 + NAA 0.01	100.00	83.33	6.33	1.50
CE10	BA 0.50 + NAA 0.10	100.00	100.00	13.67	3.50
CE11	BA 0.50 + NAA 0.50	66.67	50.00	10.33	1.17
CE12	BA 1.00	66.67	33.33	7.00	1.00
CE13	BA 1.00+ NAA 0.01	50.00	50.00	13.83	1.67
CE14	BA 1.00 + NAA 0.10	83.33	83.33	11.50	1.17
CE15	BA 1.00+ NAA 0.50	83.33	50.00	15.17	1.00
CE16	BA 3.00	100.00	100.00	7.83	1.00
CE17	BA 3.00 + NAA 0.01	66.67	66.67	6.33	1.00
CE18	BA 3.00 + NAA 0.10	100.00	83.33	11.00	1.83
CE19	BA 3.00 + NAA 0.50	100.00	100.00	9.50	1.33
CE20	BA 5.00	50.00	50.00	15.33	1.00
CE21	BA 5.00 + NAA 0.01	100.0	66.67	9.67	1.00
CE22	BA 5.00 + NAA 0.10	83.33	83.33	12.17	1.00
CE23	BA 5.00 + NAA 0.50	66.67	50.00	16.33	1.17
CE24	Kinetin 0.50	33.33	33.33	8.17	1.00
CE25	Kinetin 0.50 + NAA 0.01	50.00	33.33	11.17	1.00
CE26	Kinetin 0.50 + NAA 0.10	50.00	50.00	14.67	1.83
CE27	Kinetin 0.50 + NAA 0.50	100.00	100.00	11.67	2.00
CE28	Kinetin 1.00	66.67	66.67	8.50	2.17
CE29	Kinetin 1.00 + NAA 0.01	100.00	66.67	10.83	1.00
CE30	Kinetin 1.00 + NAA 0.10	50.00	50.00	4.33	1.17
CE31	Kinetin 1.00 + NAA 0.50	66.67	33.33	11.50	1.00
CE32	Kinetin 3.00	66.67	66.67	15.67	1.33
CE33	Kinetin 3.00 + NAA 0.01	66.67	50.00	6.50	1.17
CE34	Kinetin 3.00 + NAA 0.1	83.33	50.00	15.17	1.17
CE35	Kinetin 3.00 + NAA 0.50	66.67	66.67	9.00	1.00
CE36	Kinetin 5.00	83.33	83.33	16.67	1.00
CE37	Kinetin 5.00 + NAA 0.01	83.33	50.00	13.33	1.00
CE38	Kinetin 5.00 + NAA 0.10	50.00	50.00	10.17	1.00
CE39	Kinetin 5.00 + NAA 0.50	100.00	83.33	15.00	1.00
Control		83.33	83.33	21.67	1.00
CD(0.05)				0.98	0.36

Table 2. Effect of plant growth substances on multiple shoot proliferation of papaya.

Treatment	Plant growth substances (mg l-1)	Days for bud initiation	Number of shoots per culture	Length of longest shoot (cm)
MSP1	BA 0.00 + NAA 0.01	8.67	1.00	2.18
MSP2	BA 0.00 + NAA 0.1	5.00	1.17	1.28
MSP3	BA 0.00 + NAA 0.5	5.00	1.50	1.93
MSP4	BA 0.20	4.50	1.00	0.70
MSP5	BA 0.20 + NAA 0.01	8.17	1.00	2.15
MSP6	BA 0.20 + NAA 0.10	6.17	3.17	2.63
MSP7	BA 0.20 + NAA 0.50	3.17	1.67	1.68
MSP8	BA 0.50	4.67	2.17	2.90
MSP9	BA 0.50 + NAA 0.01	10.83	1.33	0.85
MSP10	BA 0.50 + NAA 0.10	8.67	6.50	5.78
MSP11	BA 0.50 + NAA 0.50	4.50	3.50	4.08
MSP12	BA 1.00	3.83	1.50	1.18
MSP13	BA 1.00 + NAA 0.01	7.00	1.17	2.15
MSP14	BA 1.00 + NAA 0.10	10.83	4.33	3.72
MSP15	BA 1.00 + NAA 0.50	5.33	1.00	2.05
MSP16	BA 3.00	10.83	1.50	1.53
MSP17	BA 3.00 + NAA 0.01	8.00	5.33	3.30
MSP18	BA 3.00 + NAA 0.10	12.33	1.00	0.87
MSP19	BA 3.00 + NAA 0.50	5.83	2.83	2.68
MSP20	BA 5.00	10.33	1.33	1.20
MSP21	BA 5.00 + NAA 0.01	5.83	2.67	2.05
MSP22	BA 5.00 + NAA 0.10	9.50	1.33	2.78
MSP23	BA 5.00 + NAA 0.50	12.17	1.00	1.37
MSP24	Kinetin 0.50	6.33	1.00	2.05
MSP25	Kinetin 0.50 + NAA 0.01	4.00	4.17	3.75
MSP26	Kinetin 0.50 + NAA 0.10	10.00	1.50	1.22
MSP27	Kinetin 0.50 + NAA 0.50	4.50	2.33	3.08
MSP28	Kinetin 1.00	9.67	2.17	2.22
MSP29	Kinetin 1.00 + NAA 0.01	9.67	1.00	1.05
MSP30	Kinetin 1.00 + NAA 0.10	12.83	1.00	1.40
MSP31	Kinetin 1.00 + NAA 0.50	8.50	5.33	2.07
MSP32	Kinetin 3.00	5.67	2.17	2.45
MSP33	Kinetin 3.00 + NAA 0.01	13.50	1.00	3.60
MSP34	Kinetin 3.00 + NAA 0.10	9.67	1.00	0.73
MSP35	Kinetin 3.00 + NAA 0.50	4.50	1.17	1.18
MSP36	Kinetin 5.00	10.00	1.00	1.28
MSP37	Kinetin 5.00 + NAA 0.01	11.50	1.00	2.62
MSP38	Kinetin 5.00 + NAA 0.10	8.50	1.67	1.18
MSP39	Kinetin 5.00 + NAA 0.50	6.00	1.00	3.10
Control		-	1.00	0.55
CD (0.05)		1.09	0.62	0.2

0.1 mg/l. In a similar experiment, Rohman *et al* (2007) used Kinetin 1.0 mg/l for better establishment of papaya explants. Highest bud initiation was recorded from BA 0.5 mg/l along with NAA 0.1 mg/l (Table 1). The result of the present study was in agreement with the observations of Priyakumari (2001) who observed that MS medium supplemented with BA 0.5 mg/l and NAA 0.1 mg/l gave the highest number of bud initiation in establishment medium. This may be due to the fact that BA and its combination with auxin induced more bud initiation as reported by Suthamathi *et al* (2002). Increase in level of cytokinin than auxin favoured bud initiation in papaya (Kabir *et al*, 2007). The above reports were thus in agreement with the results of the present studies.

Application of BA 0.5 mg/l along with NAA 0.1 mg/l was found to be better for shoot proliferation in papaya (Table 2). The results of the present experiment were in line with the findings of Adigo *et al* (2015) who reported that maximum number of shoots in papaya variety Rajshahi-red was produced from shoots cultured in MS medium supplemented with BA 0.5 mg/l along with NAA 0.1 mg/l. Full strength MS medium produced highest shoot proliferation rate, better survival of plantlets in papaya variety Pusa Nanha. This was in agreement with the findings of Lai *et al* (2000), who reported that MS medium at full strength was used for the micro-propagation of papaya. Similar results as suitability of full MS medium for *in vitro* propagation of papaya was also reported by Suthamathi *et al* (2002) and Usman *et al* (2002).

Sucrose 30.0 g/l in shoot proliferation medium produced maximum number of shoots and also resulted in better survival and further elongation of shoots.. These results were supported by findings of Fitch and Maureen (2003) who observed that sucrose 30.0 g/l was effective for micro-propagation of papaya. Suthamathi *et al* (2002) also reported that MS medium supplemented with sucrose 30.0 g/l increased multiple shoot proliferation in papaya.

The study revealed that agar 6.0 g/l produced highest shoot number in papaya. The addition of optimum agar concentration creates an osmotic potential favorable for the uptake of nutrients. The probable reason may be the enhanced absorption of nutrients from the media due to the lowered osmotic potential. A change in agar concentration affects the nutrients in culture media as well as over all nutrient concentration in the experiment (Protul *et al*, 2012). The results of the study showed that the highest shoot length was noticed by the addition of agar 6.5 g/l in papaya.

In the present study amino acids arginine and glycine at different concentrations were supplemented to the media, to study the shoot proliferation rate. Maximum shoot proliferation and better survival was noticed with the addition of aminoacid glycine, 100.0 mg/l (Table 3). It was observed that at higher concentrations of arginine and glycine, there was a significant reduction in

Table 3. Effect of amino acids on multiple shoot proliferation of papaya.

Treatment	Amino acids	Survival (%)	Shoots per culture	Length of longest shoot (cm)
GL1	Glycine-50.00	66.67	2.67	0.75
GL2	Glycine-100.00	100.00	3.50	2.37
GL3	Glycine-200.00	66.67	1.17	0.27
AR1	Arginine-50.00	83.33	2.33	1.87
AR2	Arginine-100.00	100.00	4.50	0.63
AR3	Arginine-200.00	100.00	2.00	0.35
Control		66.67	3.67	2.05
CD (0.05)			1.01	0.10

shoot number and shoot length. This may be due to antagonistic effect of these amino acids when the concentration exceeds optimum level.

Addition of activated charcoal 0.05 per cent (Table 4) increased shoot proliferation rate and shoot length in papaya. Activated charcoal adsorbs the toxic substances and residual cytokinin from the medium (Ming *et al*, 2007). Shoot elongation occuring by the addition of activated charcoal may be attributed to the adsorption of phytohormones on activated charcoal. The result of the present experiment was in confirmity with findings of Adigo *et al* (2015) also, who observed that activated charcoal 0.05 per cent was ideal for the *in vitro* propagation of papaya. Cobalt chloride 5.0 mg/l increased shoot proliferation rate and shoot length in papaya, while the highest survival percentage was obtained with the addition of Cobalt chloride 10.0 mg/l. Fitch and Maureen (2003) added ethylene inhibitors amino ethoxy vinyl glycine (AVG) and Cobalt chloride ($CoCl_2$) to the culture medium of papaya and the results indicated that shoot proliferation rates enhanced by 23 per cent and 49 per cent respectively. The present experiment also showed similar results. The present study revealed reduced shoot number and length of shoots in control plants. This finding was in line with the observations of Hidaka *et al* (2008) who observed that ethylene accumulation is deleterious to culture growth and development.

Light had significant influence on shoot proliferation. The trial revealed that cultures under light produced maximum number of shoots and increased shoot length in papaya. Better survival of plants was also observed under light conditions. The shoots and leaves appeared pale and chlorotic under dark conditions. This can be attributed to the lack of chlorophyll development required for photosynthesis and in turn shoot regeneration, which might have contributed to the reduced rate of shoot proliferation of cultures under dark. The positive effects of light was observed by Kabir *et al* (2007) and Protul *et al* , 2012 .

Among the various plant growth substances tried for *in vitro* rooting, full strength MS medium supplemented with IBA 2.0 mg/l induced early rooting. The above results from the present study are in line with the findings of Rogayah *et al* (2012) who reported that full strength MS medium was ideal for *in vitro* rooting of papaya. Highest number of roots was obtained by the addition of IBA 3.0 mg/l in papaya (Table 5). Anandan *et al* (2011) reported that in papaya under *in vitro* conditions, application of IBA 3.0 mg/l promoted highest number of roots. The present experiment also showed similar result. Highest number of roots was noticed with the addition of sucrose 30.0 g/l to the rooting medium. Similar studies of Fitch and Maureen (2003) showed that highest *in vitro* rooting in papaya was obtained by adding sucrose 30.0 g/l to the rooting medium. This finding was in confirmity with the observations of the present study. The beneficial effect of high sucrose concentration might be because the optimum level of sucrose for root formation depends on a balance between the sucrose and total nitrogen in the medium. Addition of activated charcoal 0.05 per cent to the rooting medium induced early rooting and highest number

Table 4. Effect of activated charcoal on multiple shoot proliferation of papaya.

Treatment	Activated charcoal (%)	Survival (%)	Shoots per culture	Length of longest shoot (cm)
AC1	0.05	100.00	5.50	1.82
AC2	0.10	83.33	3.50	0.27
AC3	0.20	83.33	1.00	0.65
Control		100.00	1.67	1.13
CD (0.05)			0.95	0.13

of root. Panjaitan *et al* (2007) reported that presence of activated charcoal stimulated rhizogenesis in papaya. Improved rooting response has been observed in many instances when activated charcoal was included in the medium (Rogayah, 2012). This might be because activated charcoal adsorbs the toxic substances and cytokinins inhibitory to rooting.

CONCLUSION

The refined tissue culture protocol of papaya revealed that apical buds and lateral buds from mature plants and seedlings could be used as explants for *in vitro* propagation. However, explant for papaya is apical bud. For surface sterilization of explants, apical buds should be treated in 0.08 per cent mercuric for ten minutes and for lateral buds it is twelve minutes. Application of BA 0.5 mg/l along with NAA 0.1 mg/l was found to be better for initial culture establishment. The culture establishment medium is useful for conditioning of the explant and for stimulating its initial growth. The study revealed that full strength MS medium supplemented with sucrose 30.0 g/l and agar 6.0 g/l, amino acid arginine 50.0 mg/l, activated charcoal 0.05 per cent, Cobalt chloride 10.0 mg/l was is ideal. For multiple shoot proliferation combined application of BA 0.5 mg/l along with NAA 0.1 mg/l was found to be the best. Cultures placed under light produced maximum number of shoots and increased shoot length in papaya. Better survival of papaya plants also was observed under light conditions. For *in vitro* rooting, full strength MS medium supplemented with IBA 3.0 mg/l and sucrose 30.0 g/l is found promising. Improved rooting response has been observed when activated charcoal 0.05 per cent was included in the medium.

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Table 5. Effect of plant growth substances on *in vitro* rooting of papaya.

Treatment	Plant growth substances (mg l-1)	Rooting (%)	Number of roots	Length of longest root (cm)
R1	IBA 0.50	66.67	-	-
R2	IBA 1.00	33.33	2.00	2.23
R3	IBA 2.00	66.67	1.17	2.70
R4	IBA 3.00	100.00	6.33	3.28
R5	IBA 4.00	-	2.50	1.13
R6	IAA 0.10	50.00	-	-
R7	IAA 0.50	83.33	1.00	0.88
R8	IAA 1.00	66.67	4.17	2.42
R9	IAA 2.00	33.33	2.33	1.60
R10	NAA 0.10	-	-	-
R11	NAA 0.50	83.33	3.17	1.37
R12	NAA 1.00	66.67	1.00	2.85
R13	NAA 2.00	83.33	1.33	2.08
R14	NAA 3.00	33.33	3.50	1.47
Control		-	-	-
CD (0.05)			0.47	0.13

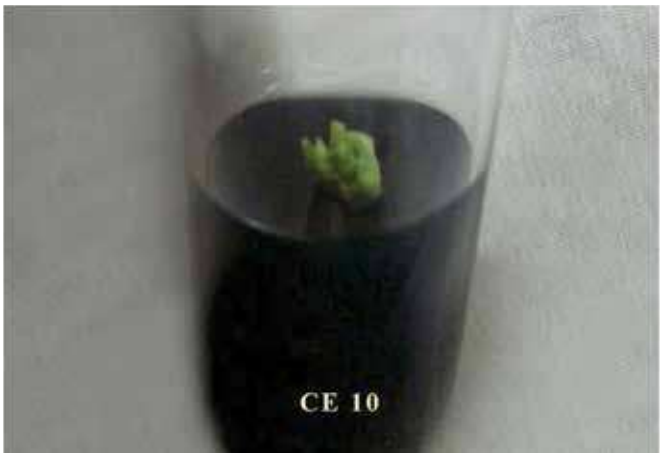

Plate 1. Bud initiation of papaya in culture establishment medium

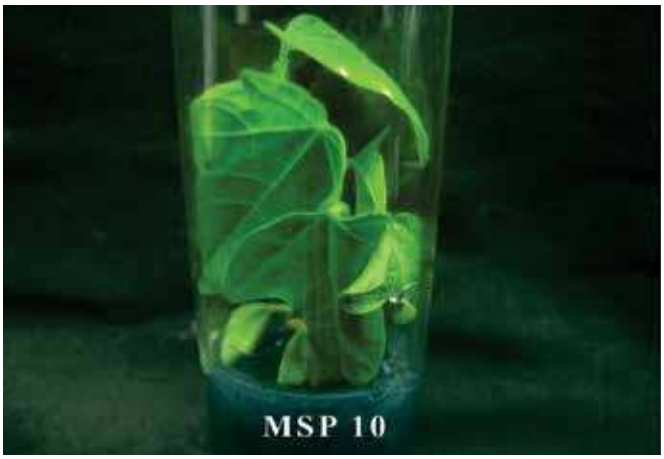

Plate 2. Multiple shoot formation of papaya in shoot proliferation medium

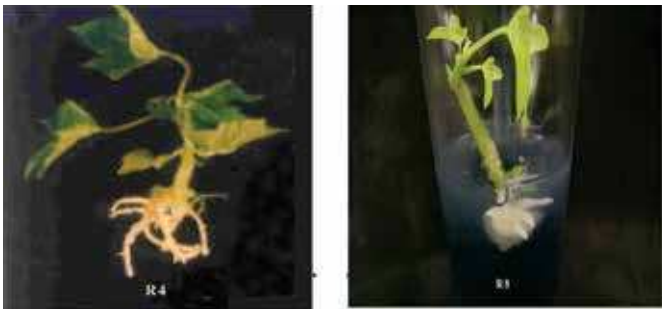

Plate 3. *In vitro* rooting of papaya

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