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MASS PROPAGATION OF SELECTED PAPAYA PLANTS BY TISSUE CULTURE

FINAL REPORT

GRANT NO : RG/AID/09

(P S T C CONTRACT 5.377)

CEYLON INSTITUTE OF SCIENTIFIC AND INDUSTRIAL RESEARCH

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ABSTRACT

Most of the papaw (Carica papaya) fruits found all over the world show considerable variation in taste, flavour, texture, keeping quality and processing characteristics. Even when selected hybrid plants are cultivated, there is significant variability in characteristics. To overcome this problem, investigations were undertaken to develop the technology for the in vitro propagation of selected papaw plants.

Apical end and axillary bud explants were used to develop the protocol for mass propagation of plants. Surface disinfested explants started growing in Murashige & Skoog (MS) basal medium containing 0.5g/L casein, hydrolyzate and 10% coconut water with the addition of 0.03 mg/L Indole Butyric acid (IBA) and 0.3 mg/L 6- Benzyl Aminopurine (BA). Shoot proliferation was most effective in this medium. Depending on the plant material five to twelve fold shoot proliferation was observed. Root formation with apical dominance occurred in MS medium supplemented with 5.0 - 6.0 mg/L IBA and 0.03 mg/L BA. Rooted plantlets were acclimatized to soil under controlled humidity, temperature and water. Growth commenced within a period of two to three weeks.

Young petiole explants produced embryonic callus after repeated subculturing in MS medium with 12.00 mg/L IBA and 0.03 mg/L BA. These embryos produced plantlets in MS medium with 6.0 mg/L IBA and 0.003 mg/L BA.

The technology developed would enable the laboratory propagation of selected papaw plants which carry desired characteristics.

1.0 INTRODUCTION

Herbaceous tropical fruit crop, Carica papaya belongs to the family Caricaceae and gives three types of plants when seed propagated. Sex determination of papaya is governed by three genetic factors, M_1, M_2 and m. The three types of plants found in nature are :

1) M_1m - Staminate flower producer. (unproductive plants)

2) M_2m - Hermaphrodite flower producer giving elongata type fruits

3) mm - Pistillate flower producer giving round fruits

All the dominant combinations are lethal. Sometimes in adverse weather conditions such as prolonging drought periods, even hermaphrodite plants also produce male flowers[1].

Papayas have conventionally been propagated from seeds and considerable variation exists in commercial plantings. Therefore tissue culture propagation of papaya offers a valuable alternative to provide genetic carbon copies of superior varieties for commercial use within a comparatively shorter period than from conventional breeding[2].

Plant tissue culture technology broadly refers to the in-vitro cultivation of all plant parts. A tissue culture system is also very often a model system which allows to investigate physiological, bio-chemical, genetic and structural problems related to plants[3].

In Sri Lanka, mosaic virus disease is a major problem in papaya cultivations. By meristem tissue culture, virus free plants can also be propagated[4].

There are two main pathways of tissue culture propagation of plants.

1. Via organogenesis - produce true to type plants
2. Via somatic embryogenesis - a) Direct
   b) Indirect - through callus where variations in plants can be observed.

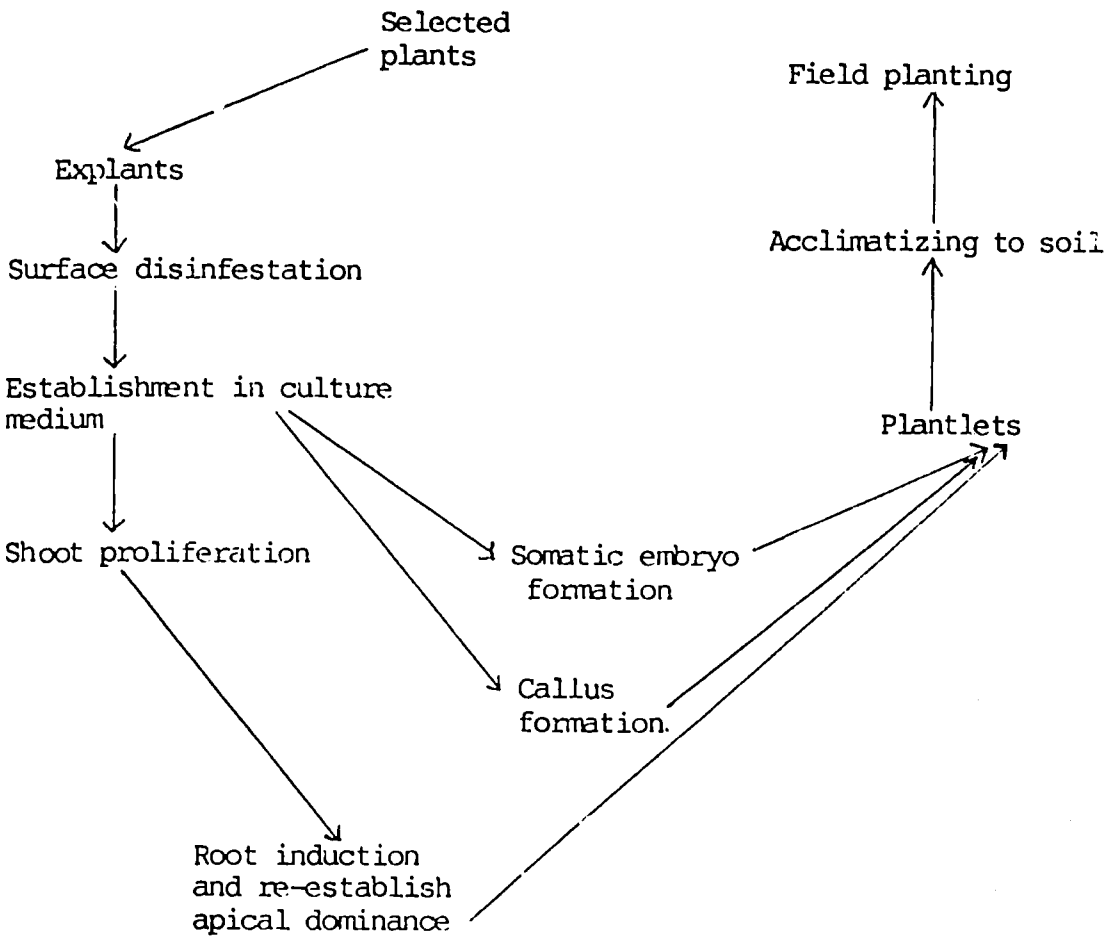

Fig. 1 - Pathways of Tissue Culture propagation of Papaya Plants.

Objectives of our study are as follows :

1. To develop the most appropriate tissue culture technology for clonal propagation of selected papaya plants.

2. To test the hypothesis of genetic variation in tissue cultured plants by somatic embyrogenesis

3. To build up expertise in modern techniques and form a team of experts to adapt tissue culture as a tool for mass production of selected commercial crops.

2.0 MATERIALS AND METHODS

2.1 Explants for culturing

Explants were taken from young seedlings and mature papaya plants bearing flowers and fruits. For organogenesis studies, plant apical end and axillary buds were taken as explants. For callus production studies, newly formed leaf petiole pieces and newly formed leaf mid rib areas were taken. Explants were taken in the morning between 8.00 a.m. to 10.00 a.m.

2.2 Media Preparation

Murashige minimal organics medium containing Murashige and Skoog plant salt mixture (MS) was used for all cultures.

Composition of the Murashige minimal organics medium

1. Murashige & Skoog plant salt mixture -

Macro elements -	mg/L
NH_4NO_3	1650.00
KNO_3	1900.00
$CaCl_2.2\ H_2O$	440.00
$MgSO_4.7\ H_2O$	370.00
KH_2PO_4	170.00

Micro elements -

Na_2 - EDTA	37.30
$FeSO_4 \cdot 7\ H_2O$	27.80
H_3BO_3	6.20
$MnSO_4 \cdot H_2O$	16.90
$ZnSO_4 \cdot 7\ H_2O$	8.60
Kl	0.83
$Na_2MoO_4 \cdot 2\ H_2O$	0.25
$CuSO_4 \cdot 5\ H_2O$	0.025
$CoCl_2 \cdot 6\ H_2O$	0.025
	4330.00

2. Sucrose 30000.00

3. 1-Inositol 100.00

4. Thiamine HCl 0.40

5. Buffering agents: Phytion TM

Basic medium composition

Murashige Minimal Organics medium	34.00 g/L
Casein hydrolyzate	0.50 g/L
Coconut water	10.00%
Agar	0.8 - 0.9%

pH of the media was adjusted to 5.7 before adding agar, and heated, in a water bath to dissolve agar. Vessels containing media were autoclaved at 15 psi (1.06 kg/cm^2) for 15 min.

MaCarthy bottles (30 ml) and Magenta flasks were used as culture vessels.

2.3 Aseptic Culturing

Handling and transfer of explants were carried out inside a Edge Gard Hood type Laminar air flow cabinet.

2.4 Surface Sterilization of explants

For disinfestation 20% clorox solution containing 1% sodium hypochlorite was used with 0.1% surfactant 'N' (wetting agent).

3.0 EXPERIMENTS

3.1 Determination of optimum time period for surface sterilization of explants

Five batches of explants each containing 30 tissues were used. Tissues were dipped in 20% clorox solution with 0.1% surfactant 'N' while shaking for different time intervals such as 4 min, 6 min, 8 min, 10 min and 12 min and washed in sterile distilled water for 3 times. Explants were cultured in Murashige and Skoog basic medium.

Number of tissues survived, bleached and contaminated were counted after 1 week.

3.2 Organogenesis studies

3.2.1 Determination of optimum growth medium for Papaya tissues

Apical end and axillary bud explants were surface sterilized and cultured in the MS basic medium with different combinations of plant hormones, auxins and cytokinins. As auxins, Napthalene acetic acid (NAA) and Indole butyric acid (IBA)

were used and as cytokinins; Kinetin (KIN) and Benzylamino-purine (BAP) were used.

Any signs of callus production and shoot initiation were noted. Cultured tissues were subcultured in every two weeks into the same medium.

3.2.2 Proliferation rate studies

Apical end and axillary bud explants were cultured in the above selected optimum growth medium. Number of new shoots formed were counted in every two week durations.

3.2.3 Determination of a suitable medium to induce rooting of Papaya shoots

With the MS basic medium different concentrations of auxins and cytokinins were used as Auxin : Cytokinin 1.

Proliferated shoots were separated aseptically and cultured in these media.

Any signs of root initiation were noted after 3 weeks.

3.2.4 Acclimatizing the rooted plants in soil

Sub soil from an uncovered place was taken and all rough particles were removed. Two batches of experiments were carried out with :

1) Sterilized soil (at 15 psi for 2 hours)
2) Non-sterile soil

8" x 6" poly bags with 3 holes at the bottom were filled with soil and used for planting.

Rooted plants in the above selected medium were taken out from the vessel and washed well to remove

any residual agar. Any callus formation at the base was also removed to avoid rotting when transferred to soil.

Washed plants were separated into 2 batches and were given two different treatments.

1. Immersed in dilute green hyponex solution for 30 min.
2. Immersed in dilute maxicrop fertilizer solution for 30 min.

Finally plants were dipped in a fungicide solution for 1-2 min and planted in soil bags. Plants were kept covered during the first two weeks in soil to avoid wilting due to sudden drop of humidity, and gradually exposed to normal field conditions.

3.2.5 Field trials on tissue culture propagated papaya plants -

Plants were kept in soil bags for one month under controlled temperature, humidity and water were transferred to the field to test morphological characteristics and the fruit quality in comparison to their mother plants. These mother plants were randomly selected from various locations. Further trials are being carried out with selected varieties of papaya plants.

3.3 Studies on somatic embryogenesis

3.3.1 Determination of a suitable medium to produce embryonic callus

Newly formed leaf petiole pieces of 0.3 - 0.5 cm in length and leaf mid rib areas were taken as explants.

Explants were surface sterilized and cultured in the MS basic medium with different levels of auxins and cytokinins.

Types of callus produced was noted after four weeks.

3.3.2 Determination of a suitable medium to generate plantlets through callus

Embryonic callus generated in the above selected medium was transferred to MS basic medium with different combinations of plant hormones. Signs of plantlet generation were noted after 10 weeks.

4.0 RESULTS AND DISCUSSION

4.1 Optimum time period for surface sterilization of explants

Dipping time of explants in 20% clorox solution (min.)	4	6	8	10	12
No. of explants cultured	30	30	30	30	30
No. of explants survived after 1 week	00	13	18	14	07
No. of contaminated explants	30	17	12	05	00
No. of bleached explants	00	00	00	11	23
% of contamination	100	56.7	40.0	16.7	00

Table 1 - The effect of dipping time in clorox solution

From the results obtained, optimum surface disinfestation time for Papaya explants was determined as 8 min. followed by three washings in sterile distilled water.

4.2 Organogenesis studies

4.2.1 Optimum growth medium for papaya tissues

Medium	Hormone concentration (mg/L)				Degree of growth after 4 weeks	
	NAA	IBA	KIN	BAP	Shoot initiation	callus production
M_1	0.2	-	-	5.0	-	+
M_2	2.0	-	-	0.2	-	+
M_3	0.5	-	-	2.0	++	++
M_4	2.0	-	0.4	-	-	+
M_5	2.0	-	10.0	-	-	+
M_6	-	5.0	0.1	-	-	+++
M_7	-	0.03	-	0.03	++	++
M_8	-	0.03	-	0.3	++++	++
M_9	-	0.03	-	10.0	++	++
M_{10}	-	0.03	-	0.05	++	+

Table 2 - The effect of different hormones and their concentrations on callus production and shoot initiation

NAA - Napthalene acetic acid

IBA - Indole 3-butyric acid

KIN - Kinetin

BAP - Benzylaminopurine

Degree of growth --- - Nil +++ Good

+ Poor ++++ Very good

++ Average

Shoot initiation was observed in M_3, M_7, M_8, M_9 and M_{10} media. Callus formation was observed in all media tested especially at cut edges. In different media callus produced were also different in nature such as spongy white, light green spongy, compact yellowish etc.

M_8 medium with 0.03 mg/L IBA and 0.3 mg/L BAP was found to be the best medium for shoot initiation and proliferation.

In M_3 medium with NAA and BAP, shoot development habit was very compacted when compared with media M_7, M_8, M_9 and M_{10} containing IBA and BAP.

It was found that the auxin IBA is more effective at a lower concentration with 10 times BAP in shoot proliferation of Papaya.

For further shoot proliferation rate studies M_8 medium was selected.

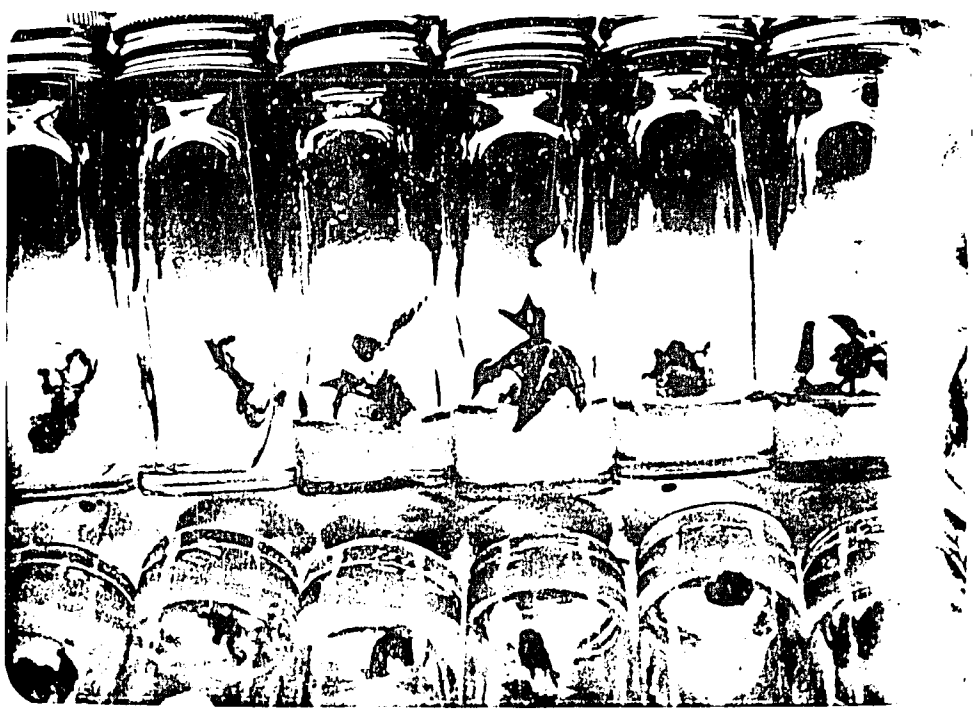

Plate 1 - Cultured explants in different media

Plate 2 - Proliferated Papaya shoots in M_8 medium

4.2.2 Proliferation Rate Studies

After 2 weeks in M_8 medium, explants gave a 5 fold increase in shoot number, and a 12 fold increase after 4 weeks.

Plate 3 - Shoot proliferation of papaya explant after 4 weeks

4.2.3 Determination of a suitable medium to induce rooting of papaya shoots

Medium	Hormone concentration (mg/L)				Observations after 3 weeks		
	NAA	IBA	KIN	BAP	Root initiation	Apical dominance	Callus production
R_1	-	0.03	-	-	N	N	✓
R_2	-	0.3	-	-	N	N	✓
R_3	-	3.0	-	-	+	N	✓
R_4	0.03	-	-	-	N	N	✓
R_5	0.1	-	-	-	N	✓	✓
R_6	0.3	-	-	-	N	N	✓
R_7	3.0	-	-	-	N	N	✓
R_8	-	5.0	-	0.03	++	✓	✓
R_9	-	6.0	-	0.03	+++	✓	✓
R_{10}	-	10.0	-	0.03	++	✓	✓
R_{11}	-	6.0	0.03	-	N	N	✓
R_{12}	-	10.0	0.03	-	N	N	✓
R_{13}	0.1	-	-	0.03	N	N	✓
R_{14}	6.0	-	-	0.03	N	N	✓
R_{15}	0.1	-	0.03	-	N	N	✓
R_{16}	6.0	-	0.03	-	N	N	✓

Table 3 - The effect of different hormones and their concentrations on root induction of papaya shoots

+ Low ✓ Present

++ Medium N Absent

+++ High

Callus formation was obvious in all the media tested. Type of callus produced and the amount varied with the hormone and its concentration. R_5 medium with 0.1 mg/L NAA was found to be the best for good apical dominance with stem elongation. But root initiation was not observed upto 4 weeks in this medium.

All the three media with IBA and BAP showed apical dominance with very low callus production when compared with other media tested. R_9 medium with 6.0 mg/L IBA and 0.03 mg/L BAP was found to be the best to produce roots within 3 weeks of culturing, with apical dominance,

R_3 medium only with 3.0 mg/L IBA and R_8 and R_{10} media with both IBA and BAP concentrations were found to be good in root initiation.

With these experiments R_9 medium was selected to produce roots in tissue cultured papaya shoots.

Plate 4 - Explants initiating roots in different media tested.

Plate 5 - Rooted plant in R_9 medium.

4.2.4 Acclimatizing the rooted plants in soil

From the two batches of soil, non-sterile soil was found to be better than sterilized soil. In sterile soil bags, fungus growth was obvious when exposed to normal field conditions.

Plant survival rate was mainly depended on the degree of washing of the plant and the amount of water applied to the plant during the first week of planting.

From the two fertilizer solutions used, no marked difference in growth of plants was obvious.

Plate 6 - Rooted Papaya plant ready to take out from the magenta flask

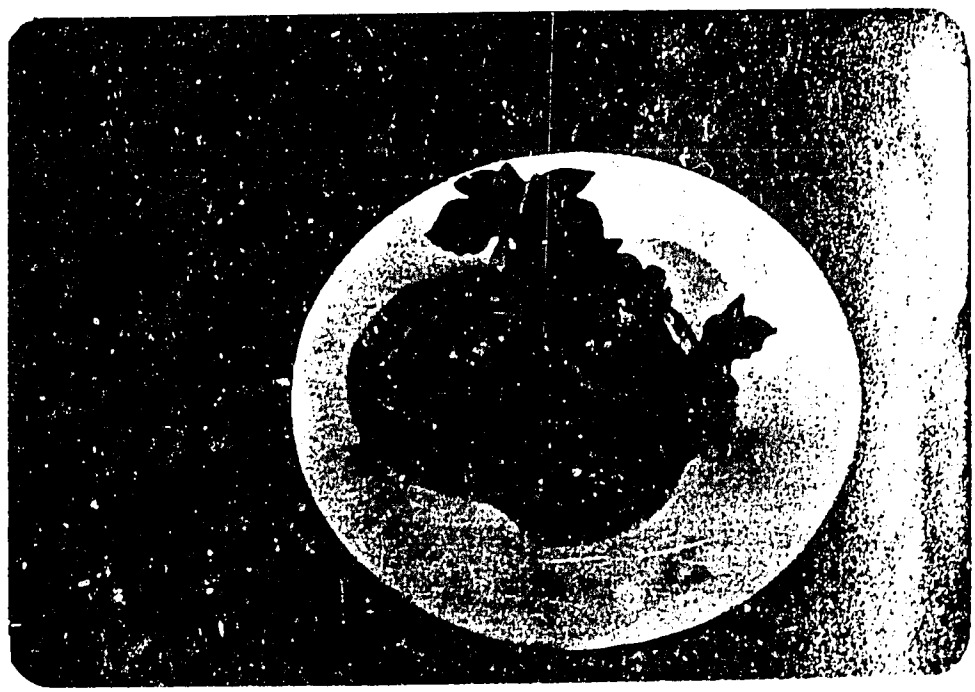

Plate 7 - Papaya plant taken out from the container and washed

It was observed that the plants having too much of roots tended to rot after planting and it was due to lack of washing. Plants nad just initiated roots and the plants in the rooting medium for 3 weeks and without having roots showed a better survival rate insoil.

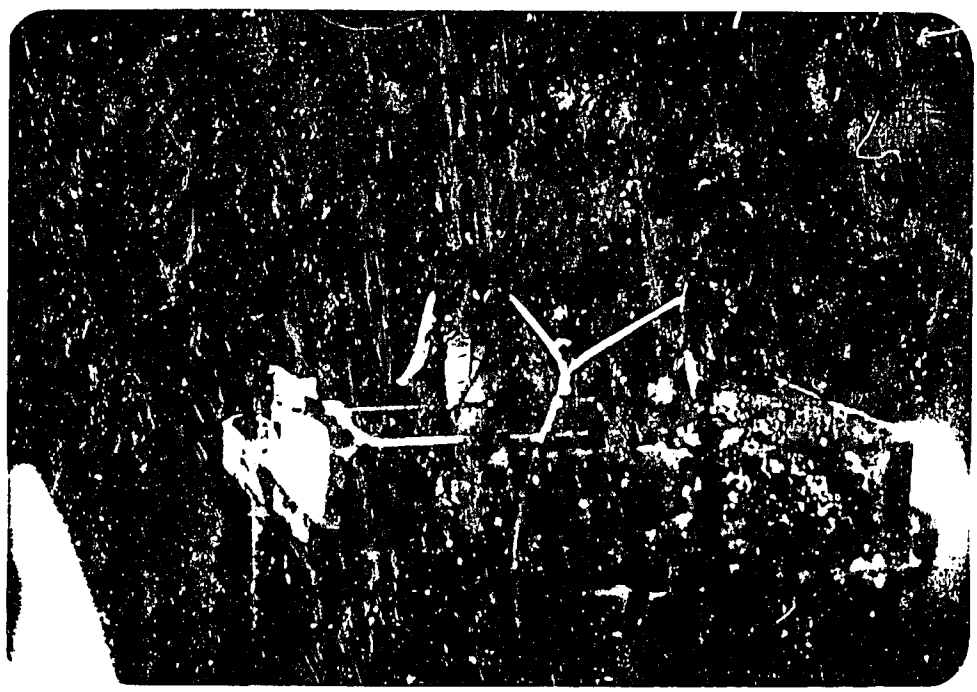

Plate 8 - Tissue cultured Papaya plant in soil

4.2.5 Field trials on tissue culture propagated papaya plants

Tissue culture propagated papaya plants and their fruits showed no morphological difference from their conventionally propagated mother plants. However few hermaphrodite plants produced male flowers under water stress conditions.

Plate 9 - Tissue culture propagated plant at the flowering stage

Plate 10 - Tissue culture propagated plant at the fruiting stage

4.3 Somatic Embryogenesis

4.3.1 Suitable medium to produce embryonic callus

In all the media tested, various types of callus were generated, but except in C_8 medium there were no signs of embryogenesis.

In C_8 medium with 12.0 mg/L IBA and 0.03 mg/L BAP, dark yellowish compact type of callus was generated and green spots were observed on the callus surface.

Type of callus and the rate of callus growth also showed a variation from explant to explant and its age.

Medium	Hormone concentration (mg/L)					Type of callus produced after 6 weeks
	2.4D	NAA	IBA	KIN	BAP	
C_1	0.05	-	-	-	-	Light green spongy
C_2	0.1	-	-	-	-	"
C_3	0.5	-	-	-	-	"
C_4	-	0.3	-	-	-	"
C_5	-	-	5.0	0.1	-	White spongy
C_6	-	-	0.3	-	-	White compact
C_7	-	-	0.03	-	-	"
C_8	-	-	12.9	-	0.03	Dark yellowish compact

Table 4 - The effect of different hormones and their concentrations on embryonic callus production from papaya explants.

Plate 9 - Callus generated in C_3 medium

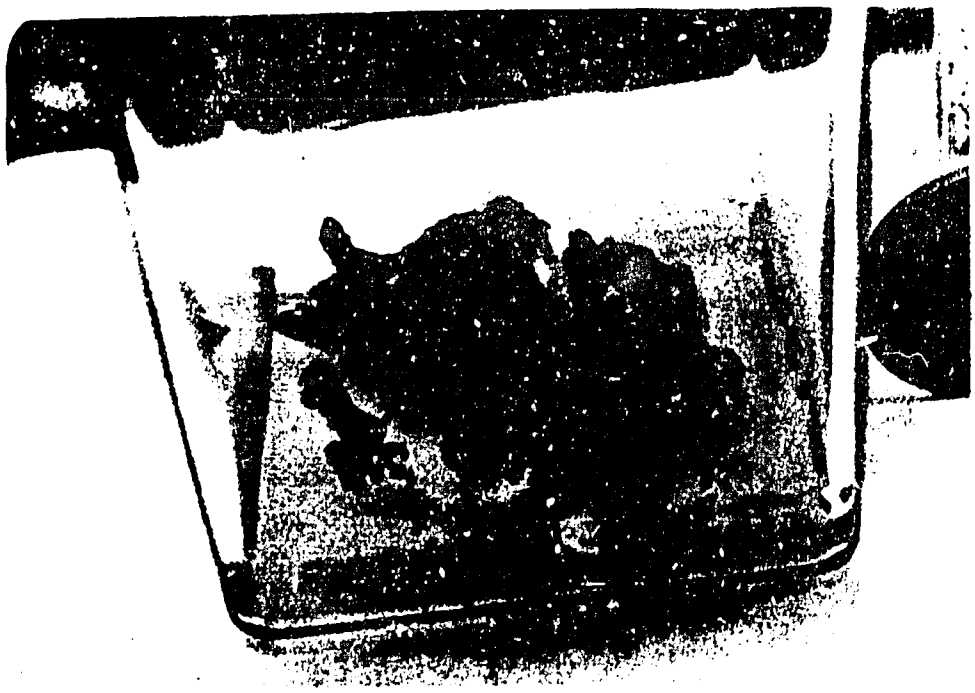

Plate 10 - Callus generated in C_4 medium

Plate 11 - Embryonic callus generated in C_8 medium

4.3.2 Suitable medium to generate plantlets through callus

Medium	Hormone concentration (mg/L)		Plant generation within 10 weeks
	IBA	BAP	
E_1	0.03	0.03	x
E_2	0.3	0.03	x
E_3	3.0	0.03	x
E_4	6.0	0.03	x
E_5	0.03	0.003	x
E_6	0.3	0.003	x
E_7	3.0	0.003	x
E_8	6.0	0.003	✓

Table 5 - The effect of different hormone levels on plantlet generation through callus

✓ present

x absent

When callus generated in C_8 medium was transferred to E_8 medium with 6.0 mg/L IBA and 0.003 mg/L BAP, plantlet generation was observed within 10 weeks after culturing. Roots of the plantlets formed a net-like structure and this became a problem when separating the plantlets which are adhering to each other.

The potential of plantlet generation was found to be more, when the regenerated callus was subcultured for about 5-6 times and maintained in-vitro for about 6-8 months.

Plate 12 - Plantlet generation through embryonic callus in E_8 medium

5.0 CONCLUSION

The experiments carried out revealed the following conclusions.

5.1 Optimum surface disinfestation time for Papaya explants of 0.5 - 1.0 cm in length was found to be 8 min in 20% clorox solution with 0.1% surfactant "N" followed by three washings in sterile distilled water.

5.2 Murashige and Skoog basal medium with 0.03 mg/L IBA and 0.3 mg/L BAP was found to be the best medium (M_8) among the tested media, which gave a 12 fold increase in shoot number after 4 weeks of culturing.

5.3 Murashige and Skoog basal medium with 6.0 mg/L IBA and 0.03 mg/L BAP was found to be the best medium (R_9) to induce rooting of papaya shoots and to re-establish apical dominance.

5.4 Growth rate of the tissues in-vitro was found to be closely related to the condition of the mother plant from which the explants were taken. To obtain a good proliferation rate, the explants should be taken from a healthy plant.

5.5 Degree of washing to remove all traces of agar before planting in soil was found to be very important to avoid fungal attacks.

5.6 To obtain a good survival rate of tissue cultured plants in soil, planting should be done in a well drained soil and water application during the first week should be very limited.

5.7 Tissue culture propagated papaya plants through organogenesis gave plantlets similar to their mother plants.

5.8 C_8 medium with 12.0 mg/L IBA and 0.03 mg/L BAP was selected as the best medium to produce embryonic callus of papaya.

5.9 E_8 medium with 6.0 mg/L IBA and 0.003 mg/L BAP was found to be the best medium to produce plantlets from embryonic callus.

5.10 Prolonged duration of subculture and aging of the callus was found to be very effective in producing papaya plantlets.

5.11 In all the experiments carried out in-vitro, seedling explants showed a higher growth potential when compared with the mature plant explants.

5.12 The main problem occured during these experiments was the higher microbial contamination in cultures. At the beginning, about 40% of explants had to be discarded and this percentage varies with the location from where the explants were taken.

6.0 ACKNOWLEDGEMENTS

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7.0 RESEARCH TEAM

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Ms. N.Y. Jayasekara - Research Assistant

Ms. M.G.D. Shiranthi - Technical Assistant

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9.0 PUBLICATIONS

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