

MICRO-PROPAGATION OF PAPAYA (*Carica papaya* L.) FROM MATURE FIELD GROWN TREES

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ABSTRACT

Shoot explants obtained from mature trees of "Clone P- 65" were cultured on MS (Murashige and Skoog, 1962) medium supplemented with 0.02 mg/l kinetin and 0.02 mg/l Indole-3-acetic acid for culture initiation. Contamination-free shoots collected from initiation medium were then transferred to different media for shoot multiplication. At an early stage, shoots responded poorly to the media. However, after 5-7 sub-cultures, slow but constant shoot growth was observed. Benzylaminopurine (0.045-0.225 mg/l) was the most effective promoter for shoot multiplication. Addition of adenine sulphate (40 mg/l) and casein hydrolysate (500-1000 mg/l) to the medium enhanced axillary shoot growth. MS medium supplemented with Benzylaminopurine (0.225 mg/l), adenine sulphate (40 mg/l), casein hydrolysate (500 mg/l), NaH₂PO₄ (170 mg/l) and riboflavin (3.76 mg/l) resulted in an improvement of shoot growth. Average of 7-10 shoots were produced per culture after 3 weeks duration. Micro-cuttings cultured on MS medium modified with 4.06 mg/l Indol-3-butyric acid and 3.76 mg/l riboflavin produced 100% rooting after an initial dark treatment of 5 days. Rooted plantlets were established in a medium consisting of soil, cattle manure and sand (2:1:1 v/v).

KEY WORDS: Axillary shoots, *Carica papaya* L., Mature field-grown trees, Micro-propagation.

INTRODUCTION

Papaya, a common fruit grown in the tropical and sub-tropical countries, is propagated conventionally from seeds and due to which a considerable variation exists in commercial plantations. Therefore, use of selected clonal materials is extremely important to ensure uniformity among the plants. However, clonal propagation methods such as grafting and rooted cuttings are slow and have limitations in large scale applications (FAO, 1979). Therefore, vegetative propagation through tissue culture technique is the only alternative method that can overcome the above limitations.

Various methods have been described for regeneration of papaya plants *in vitro* from callus (Arora and Singh, 1987; Fitch, 1993; Litz *et al.*, 1983; Pandey and Rajeevan, 1983) which may produce genetic off-types, and from apical and axillary bud explants (Cohen and Cooper, 1982; Drew, 1988; Litz and Conover, 1981). However, there are limitations for the commercial application of these techniques. Drew (1988) described the limitations of using tissue culture technique in commercial application as follows.

- i. Tissue culture of elite genotypes entails culture of buds from selected trees in the field.

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- ii. Inability to subculture indefinitely.
- iii: High level of bacterial contamination in bud explants from field grown trees.

Therefore, the present study was undertaken with a view to develop a tissue culture technique that may be helpful in the vegetative regeneration of papaya plants from explants collected from field grown elite trees.

MATERIALS AND METHODS

Disinfection of planting materials

Lateral shoots collected from three-year- field grown old female papaya (Clone-p-65) trees were washed for 30 min. in the running water and were then cut into sections so that each section contained 1-2 buds. The sections were immersed in 70% (v/v) ethanol for 10 s and subjected to different surface disinfection protocols to identify a suitable method for explant sterilization. Shoot sections were rinsed thrice in sterilized distilled water. Finally, explants were reduced in their sizes to about 5 mm in length and cultured aseptically on culture initiation medium. After 14 days, percentage of culture contamination and explant survival were recorded in respect of different disinfection protocols.

Culture Medium

For each litre of MS (Murashige and Skoog, 1962) salts, 100 mg myo-inositol, 2 mg glycine, 0.4 mg thiamine HCl, 0.5 mg pyridoxin HCl, 0.5 mg nicotinic acid, 30 g sucrose and 8 g agar were added and used as a basal medium (MSB). Medium pH was adjusted to 5.7 with 0.1 N KOH or 0.1 N HCl and autoclaved at 1.2 kg/cm² for 15 min.

Culture initiation medium

The basal medium (MSB) modified with 0.02 mg/l kinetin and 0.02 mg/

l indoleacetic acid (IAA) was used for culture initiation.

Shoot multiplication

Contaminant-free shoot bud explants collected from culture initiation medium were placed individually on 10 ml of different culture media in 2.5 x 10.0 cm test tubes with plastic caps. MSB medium supplemented with various combinations and concentrations of kinetin (KN), benzylaminopurine (BAP), indoleacetic acid (IAA), naphthaleneacetic acid (NAA), indolebutyric acid (IBA), gibberelic acid (GA₃), adenine sulphate (AS), casein hydrolysate (CH), riboflavin (RF) and NaH₂PO₄ were tested for shoot multiplication (Table 2).

One month after inoculation the cultures were sub cultured on to fresh medium with the same composition at 3-4 week intervals. After about 5-7 subculture cycles, average number of shoots greater than 10 mm in size, were recorded.

In vitro rooting

Shoots about 2-3 cm in length collected from multiplication media were transferred to different media supplemented with all possible combinations of MS salt levels (full and 1/2 strength macro MS salts with full strength MS micro salts), IBA (2.03 and 4.06 mg/l) and Riboflavin (0 and 3.76 mg/l). Thereafter, the cultures were subjected to three initial dark treatment periods (0, 5 and 10 days) before they were incubated under 16 h photoperiod. After 18 days, percentage of rooting and basal callus formation of shoots were recorded.

Culture environment

Unless otherwise mentioned all the cultures were incubated in an air-conditioned environment of 26± 2° C illuminated with fluorescent tubes for 16 hours.

Planting

Rooted micro-cuttings rinsed in tap water and dipped into fungicide solution (Thiram 125 mg/l) and transplanted directly in 4 cm diameter polythene bags containing sterilized soil mixture consisting of top soil, cattle manure and sand (2:1:1 v/v). The plantlets were maintained under high humidity conditions for approximately 1-2 weeks in the greenhouse.

RESULTS AND DISCUSSION

Disinfection of planting materials

Response of different disinfection protocols on culture contamination, explant survival and explant bleaching are summarized in Table 1. All explants dis-infected with the standard method (protocol 1) were contaminated. The principle contaminant was bacteria but fungi were also found. Increasing disinfectant (Clorox) concentration and/or prolonged exposure of explant to disinfectant solution (protocol 2 and 3) resulted in reduction of culture contamination, but the survival of explants was affected due to high concentration of Clorox. However, prolonging the exposure of explants to diluted disinfectant solution and then transferring into concentrated disinfectant solution only for a short period (protocols 4, 5 and 6) resulted in lower culture contamination as well as increased explant survival.

In vegetative propagation programmes, mature trees are generally preferred over juvenile, even though mature trees are often much more difficult to propagate vegetatively than juvenile trees (Bonga, 1981). Litz and Conover (1981) reported that the use of field grown papaya plants as experimental material for *in-vitro* studies has certain obvious limitations such as high rate of bacterial

contamination, proliferating cultures cannot be maintained indefinitely, stimulation of lateral bud growth declines sharply after 12-13 subcultures and rooting also becomes more difficult. However, if tissue culture systems are to be of immediate practical value to the plant breeder, field grown plants must be utilized. High level of bacterial contamination in bud explants from field grown trees has been reported and often cultures have to be discarded (Cohen and Cooper, 1982; Drew, 1988). Similar observations have been made in micro-propagation of papaya 'Clone P-65' in this study. However, by modification of general sterilization protocol of papaya shoots, rate of microbial contamination can be minimized.

Enhanced axillary branching and production of apically dominant shoots

All the treatments tested for shoot multiplication stimulated some growth of lateral buds, but only some treatments stimulated development of axillary branches (Table 2). Among the two cytokinins tested for shoot multiplication through axillary branching, BAP was the more effective promoter, while KN (media code SM₁) had little effect. However, at 1 mg/l BAP shoot growth was poor, but it was observed that axillary bud break and production of shoot primordia were enhanced. Addition of adenine sulphate and/or casein hydrolysate into the nutrient medium resulted in rapid shoot multiplication (Table 2). Addition of riboflavin, NaH₂PO₄, adenine sulphate and casein hydrolysate resulted in a dramatic improvement in shoot growth and multiplication (Table 2 and Fig. 1).

Yellowing of leaf and senescence have been observed in all the nutrient media after about 2-3 weeks of culturing. About 7-10 days after culturing, stimulation of leaf yellowing

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Table 1. Influence of different disinfection protocols on culture contamination, explant survival and explant bleaching of papaya clone P-65.

Protocol	Treatment	Contamination %	Survival %	Bleaching %
1	15% Clorox for 20 min.	100	0	-
2	15% Clorox for 45 min.	95	0	5
3	20% Clorox for 45 min.	20	0	80
4	0.5% Clorox for 1h. and then 15% Clorox for 20 min.	65	30	5
5	0.5% Clorox for 1 h. then treated 15% Clorox for 10 min. and finally 5% Clorox for 10 min.	75	10	15
6	0.01% Clorox for 3-4 h. then 15% Clorox for 20 min.	30	60	10

Table 2. Response of different nutrient media compositions on shoot growth and axillary shoot proliferation of papaya "Clone P-65" (after 3 weeks of culturing).

Media Code	Media description	Shoot growth	Average number of shoots greater than 10 mm in length
SM ₁	0.02 mg/l KN + 0.02 mg/l IAA + 1g/l CH	+	ND
SM ₂	1.0 mg/l BAP + 0.02 mg/l NAA	+	ND
SM ₃	0.1 mg/l BAP + 0.1 mg/l NAA + 1g/l CH	++	2-3
SM ₄	0.05 mg/l BAP + 0.4 mg/l IBA + 3.5 mg/l GA3 + 3.76 mg/l RF	+	1-2
SM ₅	0.2 mg/l BAP + 40 mg/l AS	+	1.2
SM ₆	0.2 mg/l BAP + 40 mg/l AS + 1g/l CH	++	3.5
MS ₇	0.2 mg/l BAP + 40 mg/l AS + 170 mg/l NaH ₂ PO ₄ + 500 mg/l CH + 3.76 mg/l RF	+++	7-10

+	-	fair	+++	-	excellent
++	-	good	ND	-	Not Determined



Fig.1. Proliferating papaya shoots in the medium supplemented with riboflavin, NaH_2PO_4 , adenine sulphate and casein hydrolysate.

and senescence have been observed in a nutrient medium that was supplemented with gibberelic acid (media code SM_5).

A two-step system, culture initiation or establishment followed by multiple shoot initiation has been found to be necessary in shoot tip culture of papaya for optimal results. A similar two-step procedure has also been practiced by Drew (1988) and Litz and Conover (1981).

In axillary shoot proliferation, a cytokinin is utilized to overcome the apical dominance in shoots and to enhance the branching of lateral buds from leaf axils. In general, BAP is the most effective cytokinin for stimulating shoot proliferation (Litz and Conover, 1981) and therefore, different concentrations of BAP have been used in this study.

Supplementing nutrient medium with additives such as adenine sulphate and casein hydrolysate are very common in tissue culture techniques and these additives have shown

varying effects on shoot formation. Cytokinins are adenine derivatives, and therefore, adenine acts as a cytokinin precursor. Because of this reason, adenine sulphate has been used as a supplement in culture media for shoot multiplication. Cohen and Cooper (1982) reported that supplementing with adenine sulphate and casein hydrolysate resulted in rapid growth of axillary shoots.

In papaya micropropagation it has been observed that in early stages of multiplication cycles (each at 3-4 week intervals), shoots poorly responded to culture medium (Table 2). However, after 5-7 subculture cycles in the same medium it was slow but shoot growth was constant. This may be due to rejuvenation of cultured shoots by repeated subculture. Bonga (1981) reported that repeated grafting and or repeated subculture of successive generations of buds resulted in partial rejuvenation of mature material. Franclet (1980) used these methods to rejuvenate *Pseudotsuga menziesii* and *Sequoia sempervirens*.

Rooting of proliferated shoots

The response of papaya shoots to different culture media and culture conditions for adventitious root formation is summarized in Table 3.

Effect of MS salts and IBA on rooting

In rooting papaya shoots responded to either both MS salts levels or IBA concentrations tested, but maximum rooting percentage (80%) was recorded in the medium based on 1/2 MS salts supplemented with 2.03 mg/l IBA. However, excellent basal callus development was observed in the cultured shoots whenever the culture medium was supplemented with only IBA.

Effect of Riboflavin and initial dark treatments on rooting

Irrespective of level of IBA, MS salts, and initial dark-treatments, nutrient media

supplemented with riboflavin resulted in absence of basal callus formation of shoots. However, when cultures were not subjected to initial dark-treatment, percentage of rooting was affected in the presence of riboflavin. Dark-treatment for the initial five days induced rooting from 40% to 100% in media supplemented with riboflavin and the maximum rooting percentage (100%) was recorded in the MS basal medium supplemented with 4.06 mg/l IBA and 3.76 mg/l riboflavin. When the duration of dark treatment was increased from 5 to 10 days, the rooting percentage decreased from 60% to 10% (Table 3).

All the shoots developed roots within 2-3 weeks. By the forth week all the plantlets had well-developed root systems (Fig. 2).

Dark incubation during auxin treatment was critical for papaya shoots. The promotive effect of darkness had been previously observed (Encina *et al.*, 1994) in *Annona*

Table 3. Influence of different levels of MS salts, IBA and Riboflavin and initial dark treatments on percentage of rooting and basal callus development of papaya "Clone P-65" micro-cuttings after 18 days in culture.

Nutrient media composition	Initial dark treatment, number of days					
	0		5		10	
	Rooting %	Basal callus	Rooting %	Basal callus	Rooting %	Basal callus
MS +2.03 mg/l IBA	40	+++	20	+++	20	+++
MS+4.06 mg/l IBA	40	+++	00	++	00	+++
1/2 MS + 2.03 mg/l IBA	80	+++	40	+++	20	+++
1/2 MS + 4.06 mg/l IBA	00	+++	40	+++	40	+++
MS + 2.03 mg/l IBA + 3.76 mg/l RF	00	-	00	-	00	-
MS + 4.06 mg/l IBA + 3.76 mg/l RF	00	-	100	-	60	-
1/2 MS + 2.03 mg/l IBA + 3.76 mg/l RF	00	-	60	-	40	-
1/2 MS + 4.06 mg/l IBA + 3.76 mg/l RF	20	-	40	-	00	-

Basal callus development rank

- - No callus
 ++ - fair amount of callus
 +++ - excellent callus

cherimola. Light seems to affect formation of growth inhibitors which counteract the effects of auxin (Hasegawa *et al.*, 1989).

Drew *et al.* (1993) reported that good root initiation and root and shoot growth were observed when papaya shoots were incubated for 2 days in darkness on a medium containing IBA and riboflavin. Similar observations have been made in micropropagation of papaya 'Clone P-65' in this study.

Establishment of plantlets in soil

Tissue cultured plantlets are very susceptible to shoot and root rots and as a result sometimes 100% of plantlets may be lost. However, by using proper management practices such as regular spraying of fungicides (Thiram) and proper water management, these losses could be minimized to around 20-25% and, about 70-80% of plantlets can be established successfully in a sterilized soil mixture (soil, cattle manure, and sand=2:1:1 v/v) under high humidity conditions (Fig. 3). Well-grown plants that



Fig.2. Rooted papaya shoots.

have been propagated by using these techniques were established in the field at HORDI, Gannoruwa (Fig.4).

CONCLUSIONS

Results clearly indicate the possibility of propagating mature elite trees by the tissue culture technique. There is hope that the tissue culture technique could be used for mass production of papaya plantlets.



Fig. 3. Acclimatization of papaya plantlets.



Fig. 4. Papaya tree (clone P- 65) established at HORDI field, Gannoruwa.

There is a chance for high rate of microbial contamination during the culture initiation, probably that can be overcome by proper mother plant management and by adapting a general sterilization procedure during culture initiation. Papaya shoots responded well to BAP at lower concentrations and apically dominant proliferated shoots can be induced through repeated subculture. Initial dark treatment and riboflavin showed a beneficial effect on rhizogenesis. Plantlets propagated by the tissue culture method can be easily acclimatized successfully in the soil mixture and later they can be planted in the field.

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