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A revised protocol for *in vitro* propagation of *Carica papaya* using lateral buds from field-grown trees

Prakash Patil, Neeta Vastrad, M. R. Dinesh and A. R. Bantwal[1]
Indian Institute of Horticultural Research
Hessarghatta Lake post, Bangalore-560 089, India
E-mail: pnp@iihr.ernet.in

ABSTRACT

A revised protocol has been developed for *in vitro* propogation of papaya using explants from field-grown trees. Successful establishment of papaya *in vitro* using lateral buds could be obtained by treating the buds with Carbendazim (0.2%) and Streptomycin (0.1%) for 24h, followed by surface sterilization with mercuric chloride (0.1%) for 3 minutes and culturing on MS medium supplemented with BAP (0.3 mg/l) and NAA (0.1 mg/l). Established buds were proliferated on modified MS medium supplemented with BAP (0.3 mg/l) and NAA (0.1 mg/l). Modified MS medium supplemented with BAP (0.3 mg/l), NAA (0.1 mg/l) and GA_3 (1 mg/l) caused extensive elongation of shoots. Elongated shootlets were rooted on half-strength MS medium supplemented with BAP (0.1mg/l), NAA (0.1 mg/l) and IBA (2 mg/l). Rooted plantlets were initially hardened on a potting mixture consisting of soilrite and later on a mixture of sand, soil and FYM (1:1:1).

Key words: Micropropagation, mature explants, *Carica papaya*

INTRODUCTION

Papaya, being a highly cross-pollinated crop, is polygamous in nature when propagated through seeds. It is cultivated worldwide using dioecious cultivars in the sub-tropical region and with gynodioecious cultivars in the tropical region, which segregate into female and hermaphrodite offspring. In commercial cultivation, one third of the females in a gynodioecious population need to be removed as these have limited economic value. Dioecious varieties normally produce 50% male plants, if propagated by seed. In addition, the papaya ring spot virus (PSRV) is a major disease in papaya causing 70-80% loss in plantations. Though this can be overcome using resistant varieties, these would lose their resistance if propagated by seeds. These problems however, can be solved if the plants are clonally propagated.

Clonal propagation through *in vitro* methods of known sex types is a better option since conventional techniques like use of cuttings and grafting have resulted in limited success. Papaya, being polygamous, requires that the explants be excised from a known sex type, which can be realised only when the tree attains reproductive maturity. Thus sex determination in papaya plants at the seedling stage or selecting explants from the mature tree enables propagation of the known sex. Successful true-to-type propagation under *in vitro* conditions can be achieved if explants are taken from mature, field-grown trees. Studies on use of lateral buds from field-grown trees have been successful under *in vitro* conditions but commercial exploitation on large scale remains unexploited due to lack of a micropropagation protocol. Hence, clonal propagation of individuals of known sex can be successfully applied to true-to-type propagation of *Carica papaya.*

MATERIAL AND METHODS

Explant preparation

Axillary buds were dissected from nodes of field-grown hermaphrodite, bearing plants of var. Surya in plastic covers and kept under running water with 1-2 drops of Tween-20 for 2h to minimize the flow of latex. These explants of 4-5mm size were pre-treated with carbendazim (0.2%) and Streptomycin (0.1%) for 24h on a shaker at 150 rpm, followed by surface-sterilization with mercuric chloride (0.1%) for 3 min. The explants were rinsed 4-5 times in sterile distilled water to wash off residual sterilants and were then inoculated on Medium. In all the experiments 20 explants were taken and replicated three times.

[1]Department of Biotechnology, Kuvempu University, Shankaraghatta - 577451

Media and culture conditions

Explant establishment

Treated explants were inoculated onto Murashige and Skoog (1962) basal medium supplemented with different concentrations and combinations of cytokinins viz., BAP (0.2, 0.3, 0.5, 2.5 mg/l) and kinetin (2.5mg/l) and auxins NAA (0.1, 0.2, 0.5 mg/l), IAA (0.175, 0.2, 0.5, 1.0 mg/l). Media were gelled with 0.8% agar. pH of the media was adjusted to 5.8 prior to autoclaving at 103.4 kPa for 20 min.

Subculture for proliferation and elongation

Contamination-free cultures were sub-cultured onto establishment medium at every 15 days. The establishment medium comprised of Murashige and Skoog (1962) basal medium supplemented with various concentrations and combinations of plant growth regulators (NAA at 0.1, 0.2 and 0.5mg/l, IAA at 0.175, 0.2, 0.5 and 1.0mg/l, BAP at 0.2, 0.3, 0.5 and 2.5mg/l and Kinetin at 2.5mg/l). The same medium was used for proliferation of explants.

When the shootlets were nearly 2mm in length, they were transferred to elongation medium containing MS basal salts with BAP (0.3mg/l), NAA (0.1mg/l) and Gibberellic acid (GA_3) (0.5, 1.0 and 2.0mg/l).

Subculture for rooting

Well-developed shoots (3-4 cm long) were then transferred onto rooting medium to induce rhizogenesis under *in vitro* conditions. To promote *in vitro* rhizogenesis, ¾, ½, and full strength Murashige and Skoog (1962) basal medium supplemented with different concentrations and combinations of plant growth regulators (IBA at 0.5, 1.0 and 2.0mg/1, NAA at 0.1mg/l and BAP at 0.1mg/l) were used.

Acclimatization

Well-developed shootlets of *Carica papaya* with *in vitro*-formed roots were removed from culture media and transplanted into netted pots containing Soilrite™. These were maintained at 90% relative humidity by covering with polythene. Later, holes were punched on these covers to permit transpiration. During the hardening period, temperature of 25±1°C and 16h photoperiod was maintained. The *in vitro* hardened *Carica papaya* plantlets were further hardened under *ex vitro* conditions with sterilised FYM: sand: soil mixture in the ratio of 1:1:1. Subsequently, these primary hardened plants were transferred (at 1½ months) to greenhouse conditions and maintained there for further field-planting.

Culture incubation

Cultures were incubated at 16h photoperiod, at 25±1°C under white cool fluorescent light having an intensity of 30limol/m²/sec.

RESULTS AND DISCUSSION

Effect of 6-benzyl amino purine on shoot proliferation

In the present investigation (Table 1), explants were cultured on MS basal medium supplemented with NAA at 0.1 mg/l and different concentrations of BAP (0.1, 0.2 and 0.5 mg/l). Inclusion of BAP at 0.3 mg/l recorded the highest proliferation of 71 and 85% at 7 and 15 DAI, respectively, with low callusing. Higher concentration of BAP (0.5 mg/l) recorded a proliferation of 71% both at 7 and 15 DAI, with moderate callusing at the base of the explants. These results are contrary to the findings of Litz and Conover (1978),

Table 1. Effect of different concentrations of BAP on proliferation of papaya cultures

Type of proliferation at 7 DAI**			
Proliferation category*	BAP at 0.2 mg/l	BAP at 0.3 mg/l	BAP at 0.5 mg/l
VGP	14%	7%	0%
GP	0%	35%	7%
P	22%	43%	64%
NP	64%	7%	14%
NR	Nil	8%	15%
Type of proliferation at 15 DAI			
	BAP at 0.2 mg/l	BAP at 0.3 mg/l	BAP at 0.5 mg/l
VGP	14%	14%	0%
GP	7%	29%	7%
P	22%	36%	64%
NP	29%	7%	14%
NR	28%	14%	15%

* VGP- Very good proliferation, GP- Good proliferation, P- Proliferation, NP- No proliferation, NR-No response
** DAI- Days After Inoculation

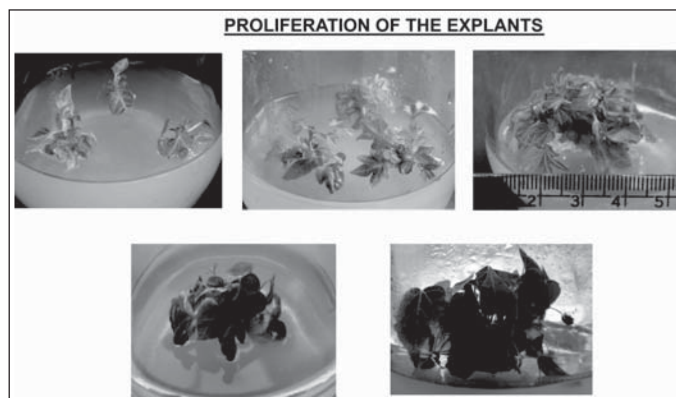

Fig 1. Proliferation of the cultures on MS medium supplemented with BAP(0.3mg/l) and NAA (0.1mg/l)

Reuveni *et al* (1990) and Drew (1988) who recorded higher multiplication rate with lowest callus production on Murashige and Skoog (1962) medium supplemented with BAP at 0.5mg/l and NAA at 0.1mg/l. In the present study, an average of five-fold increase (Fig 1) was observed upto 10 subcultures and this remained static thereafter, which is in accordance with the findings of De Winnaar (1988) who obtained a 7-fold increase in each subculture until eight cycles and then became static. Litz and Conover (1978) too observed a 7-fold increase in plant number during every cycle and cultures continued to proliferate even after the 8th subculture. Varied response of explants, in the present study, to multiple shoot proliferation may be due to the plant species, clone, physiological state of the explants, endogenous, status of cytokinins and source of the chemicals.

Effect of GA_3 on shoot elongation at different intervals

GA_3 is known to cause elongation of shoots when applied as a supplement in the medium. In the present study (Table 2), explants were cultured on MS basal medium supplemented with BAP at 0.3 mg/l, NAA at 0.1 mg/l and varying concentrations of GA_3 (0.5, 1.0 and 1.5 mg/l) for elongation of the shootlets. Tufts of the proliferated, multiple shoots were transferred onto the elongation medium after observing maximum proliferation. Results revealed that inclusion of GA_3 at 0.5 mg/l and 1 mg/l gave maximum elongation of shoots (shoot length of 2 cm) (Fig 2). De Winnaar (1988) used GA_3 in the proliferating medium which induced shoot elongation although it reduced the multiplication rate. Results in the present study are similar to the findings of De Winnaar (1988) wherein multiplication rate was lower on elongation medium compared to that in proliferation medium (Table 3). Results obtained by Reuveni *et al* (1990) are contrary to the present research findings wherein GA_3 did not have any significant effect when used for elongation of shootlets. Elongation of shootlets was also observed after prolonged culture in rooting media in papaya (Siddique *et al*, 1999).

Table 2. Effect of different concentrations of GA3 on shoot elongation in papaya shoot buds under in vitro conditions

GA3 concentration (mg/l)	VLE	E	NE	NR
Shoot elongation* at 15DAI**				
0.5	36%	14%	50%	-
1.0	36%	50%	14%	-
2.0	43%	14%	36%	7%
Shoot elongation at 30DAI				
0.5	35%	21%	43%	-
1.0	29%	64%	7%	-
2.0	21%	50%	29%	-

* E – Elongated, VLE – Very little elongation

**DAI- Days After Inoculation

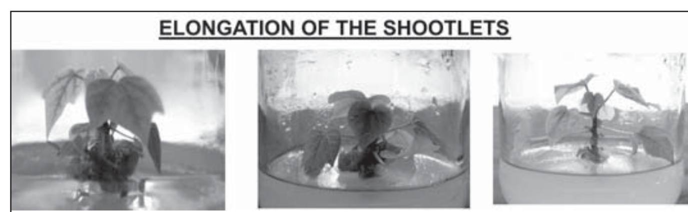

Fig 2. Elongation of shootlets on MS medium supplemented with BAP(0.3mg/l),NAA(0.1mg/l) and GA3(1mg/l)

Effect of basal medium on per cent root induction

A reduced mineral concentration in the medium increases the root initiation as reported by Drew (1987). In

Table 3. Mean multiplication rate per subculture of papaya shoots at different stages of subculture

Stage of subculture	Multiplication rate per culture cycle	
1	4.02	Subcultured on proliferation medium without GA_3
2	5.26	
3	6.69	
4	7.02	
5	4.27	Subcultured on proliferation medium containing GA_3
6	4.47	
7	5.23	
8	6.14	
9	6.32	
10	6.26	
Mean	5.57	
SEm±	0.176	
CD *(P=0.01)*	0.659	

Table 4. Effect of strength of basal medium on root initiation

Treatment	Per cent root induction	Mean number of roots per shoot	Mean root length of roots (cm)	Mean number of secondary roots (scoring)
MS	20	1.990	4.316	2.160
½ MS	45	3.800	3.075	4.710
¾ MS	37	1.740	2.699	2.030
S Em±		0.134	0.117	0.124
CD *(P=0.05)*		0.391	0.341	0.362
CD *(P=0.05)*		0.528	0.461	0.489

Number of replications per treatment = 10

Number of secondary roots	Scoring
0	0
1-5	1
6-10	2
11-15	3
16-20	4
21-25	5
26-30	6

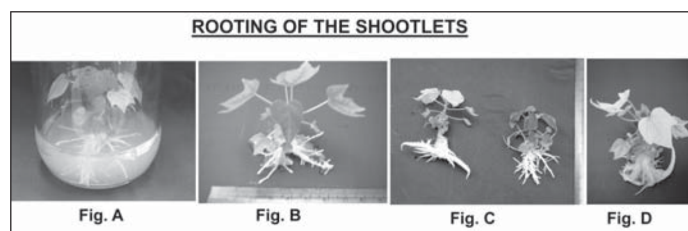

Fig 3. A-D: Rooting of the shootlets A-B: Rooting of the shootlets on ½ MS Supplemented with BAP(0.1mg/l), NAA(0.1mg/l) and IBA (2mg/l). C-D: Nature of roots grown on ½ MS Supplemented different concentrations of IBA

the present study (Table 4) different levels of Murashige and Skoog basal medium viz., full MS, ½ MS, ¾ MS were tried along with BAP at 0.1mg/l and NAA at 0.1mg/l. Culturing on ½ MS proved to induce higher percentage of root induction (45%) compared to ¾ MS (37%) and full MS (20%)(Fig 3).

The results are contrary to the findings of Teo and Chan (1994) who obtained 33% of rooting on MS medium and 26% of rooting on ½ strength MS indicating lesser percentage of root induction on reduced mineral salts (½ MS) than the normal medium (MS). Results of the present investigation revealed that reduced mineral concentration increased root initiation (45%) as against 20% on full MS thus indicating the favourable influence of reduced salt concentration on root induction. Bonga (1982) also reported that, reduction in mineral concentration has the influence on root number and initiation with tissue culture of tree species. However, Drew (1987, 1988) obtained only 30% rooting of shoots derived from mature tissue while 90% of those from 6-month-old plants within 3 weeks indicating the influence of explant age on rooting. Drew (1987) reported maximum rooting (68%) on cultures with only distilled water with 1% agar.

Effect of different concentrations of IBA on rooting

Experiments involving IBA using ½ MS basal along with BAP at 0.1mg/l and NAA at 0.1mg/l supplemented with different concentrations of IBA (0.5, 1.0 and 2.0mg/l) were tried to increase the rooting efficiency (Table 5). Best rooting (48%) of cultured shoots was achieved with ½ strength MS supplemented with BAP at 0.1mg/l, NAA at 0.1mg/l and IBA at 2mg/l. Plants on this treatment initiated more roots per plants and had better quality root system than those on IBA treatment at 0.5mg/l or 1.0 mg/l (Fig.3). Drew (1987) reported that using IBA at 2mg/l in the medium promoted good rooting of shoots in papaya. Higher concentrations of IBA and NAA in the medium caused abnormal root formation. Difference in quality of root system on plants grown on IBA and NAA

Table 5. Effect of different concentration of IBA on rooting

Treatment (I BA)	Per cent rooting	Mean number of roots per shoot	Mean root length (cm)	Mean number of secondary roots (scoring)	Nature of the root
0.5 mg/l	23	0.640	1.085	1.270	Thick blunted root
1.0mg/l	35	1.290	1.657	1.410	Thick long root with less number of secondary roots
2.0 mg/l	48	3.380	3.351	3.890	Thin, long and higher number of secondary roots
SEm±		0.133	0.113	0.126	
CD *(P=0.05)*		0.386	0.329	0.366	
CD *(P=0.01)*		0.521	0.445	0.494	

Number of replications per treatment = 10

has been also observed in grapevine and camellias (Novak and Juvova, 1982; Samautin *et al*, 1986).

In the present study, 48 % rooting was observed in plantlets Drew (1988) reported 90% rooting. However, the reason for low percentage of rooting may be light intensity, maintained at 130μ mol/m²/sec with 16h photoperiod throughout the growth period which recorded an inhibitory effect on root induction. Drew (1987) reported the use of 80μ mol/m²/sec during the root induction. However after the root initiation the growth increased as the light on the foliage increased (Drew, 1987).

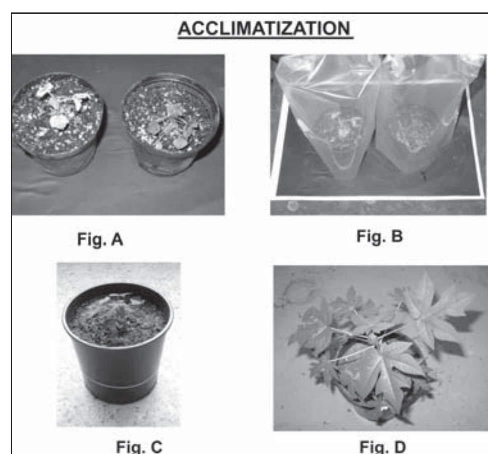

Fig 4. A-D: A-B: Primary hardening of the plantlets developed *in vitro*. C-D: Secondary hardening of the plants developed *in vitro*

Acclimatization

Acclimatization of well-developed plantlets of *Carica papaya* with *in vitro* formed shoots and roots was achieved on transplantation into netted pots containing soilrite (Fig 4). These plantlets were hardened under *ex vitro* conditions with sterilized FYM: sand: soil mixture in the ratio of 1:1:1. Later, these primary hardened plants were transferred (at 1 ½ months) to greenhouse conditions (Fig 4). Success rate for acclimatization during this stage was 90% and when plantlets were transferred to the field, all were established (Fig 5). These plants grew well and produced fruits. Similarly, Hari Prakash *et al* (1996) could harden *in vitro* generated plantlets of guava in the same combination of potting mixture (sand: soil: FYM) in the ratio of 1:1:1. Hazarika *et al* (1998) could harden *in vitro* developed plantlets of citrus by loosening the caps after 4-6 weeks of rooting. Later, these primary hardened plantlets were transplanted into a mist-house indicating the need of the plantlets for gradual change in relative humidity and temperature during acclimatization, in the present investigation.

Fig. 5 : Tissue cultured plants flowering in the field

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