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In vitro organogenesis and plantlet regeneration of (*Carica papaya* L.)

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An efficient protocol was developed for micro-propagation of Indian papaya (*Carica papaya* L.) var. Co7. Shoot bud induction occurred on culturing epicotyl segments in MS (Murashige and Skoog, 1962) medium, followed by transfer onto shoot multiplication, elongation and rooting media. Adventitious buds were induced on MS medium supplemented with varying concentration of thidiazuron (TDZ: 0.5-10 µM). The highest shoot regeneration frequency and maximum number of shoots per explant was occured on MS medium amended with 2.5 µM TDZ. The explants with initiated shoot buds produced multiple shoots in MS medium supplemented with 5.0 µM 6-benzylaminopurine (BAP) and 0.05 µM naphthaleneacetic acid (NAA). Maximum shoot elongation was observed at 1.5 µM gibberlic acid (GA_3). Upto 60.25% of the regenerated (elongated) shoots were rooted on MS medium containing 2.5 µM indole-3-butyric acid (IBA) within 3 weeks after subculturing. The *in vitro*-raised plantlets were successfully hardened first under culture room conditions, then in greenhouse with 72% survival rate. The identified regeneration system could be efficiently used in various *in vitro* manipulation studies in papaya.

Key words: *Carica papaya*, Epicotyl segments, Thidiazuron

Introduction

Papaya (*Carica papaya* L.) is an important fruit crop grown in the tropical and sub-tropical regions of the world. Major papaya producing countries are Brazil, Mexico, Nigeria, Indonesia, India, Ethiopia, Congo, Peru, China and the Philippines. It is a good source of iron, calcium, vitamins A, B and has high vitamin C. The latex of the papaya is the source of two proteolytic enzymes, papain and chymopapain (Bhattacharya and Khuspe, 2001). Botanically, Papaya plant has three different sex types: male plant producing staminate

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flower, female plant producing pistillate flower and hermaphrodite plant producing bisexual flower.

Papaya is conventionally propagated by seed for commercial plantations (Fernando *et al*. 2001). However its cultivation is hindered by problems due to the inherent heterozygosity and dioecious nature (Rajeevan and Pandey 1986; Chen *et al.,* 1987; Fitch 1993; Yang *et al*., 1996; Bhattacharya and Khuspe 2001); production of non-true-to-types (Panjaitan *et al*., 2007; Tsai *et al*., 2009); seeds of open-pollinated flowers exhibit considerable variation in shape, size and flavour (Drew and Smith 1986) and susceptibility to papaya ring spot virus (Chen *et al*., 1987; Fitch 1993; Yang *et al.,* 1996; Bhattacharya and Khuspe 2001; Clarindo *et al*., 2008; Tsai *et al*., 2009).

Efficient micropropagation of papaya has become crucial for the multiplication of specific sex types of papaya and in the application of genetic transformation technologies (Lai *et al*., 2000). Significant progress has been achieved using organogenesis and somatic embryogenesis (Yu *et al*., 2003; Litz and Conover, 1982; Reuveni *et al*., 1990; Bhattacharya *et al*., 2003; Cabral *et al*., 2008). Moreover in all these cases, shoot development was reported to have been accompanied by intervening callus phase. Callus formation is an undesirable feature during micropropagation which leads to genetic variability of regenerated plants (D'Amato, 1977). To our knowledge, there is no report on repeatable *in vitro* adventitious shoot formation the epicotyl segments as explants of papaya. In the present investigation, we have taken epicotyl segments of an Indian papaya cultivar Co7 as explants, an efficient multiple shoot induction was achieved via direct oraganogenesis from *in vitro* grown papaya seedling plants. We assure this procedure generates only true to type plantlets, which could be extended for other papaya cultivars.

Materials and methods

Plant material and explant source.

The mature seeds of *Carica papaya* var. Co7 were obtained from the Horticulture College and Research Institute, TamilNadu Agricultural University, Coimbatore India. Seeds were washed thoroughly in running tap water for 30 min. These seeds were surface disinfected with an aqueous solution of 0.1% (w/v) freshly prepared $HgCl_2$ for two min, and finally rinsed five times with sterile distilled water. The surface-disinfected seeds were germinated aseptically in half-strength MS medium with 3% (w/v) sucrose (Qualigens, India) and 0.7% agar (bacteriological grade, Hi-media, India) without exogenous supply of growth regulators.

Culture Conditions

All the media were fortified with 30 g^{-1} sucrose (Sigma- Aldrich, USA) and gelled with 0.7% agar (Sigma-Aldrich,India), The pH of the medium was adjusted to 5.8 by 1 N NaOH or 1 N HCl. The media were steam sterilized in an autoclave under 1.5 kg/cm^2 and 121°C for 15 min. All the cultures were grown at 25±2°C under a 16-h photoperiod supplied by two Philips TL 40W fluorescent tubes, and 55±5% of relative humidity

Adventitious shoot induction

In vitro-grown seedlings (10, 12, 14, 16 days after germination) were used as the source of epicotyl segments. Epicotyl segments of 3-5 mm length were aseptically removed, and cultured in sterile disposable petri dishes. In order to induce shoot buds along the cut ends, epicotyls segments were placed horizontally on shoot induction medium [MS medium (Murashige and Skoog, 1962) supplemented with varying concentrations (0.5, 2.5, 5.0, 7.5, and 10 µM of TDZ]. After 4-6 wk of culturing, the frequency of explants producing shoot and the average number of shoots per explants were recorded. All the cultures were transferred to fresh medium after every 15 days. MS basal medium without exogenous supply of growth regulators was used as control.

Shoot proliferation and elongation

To facilitate multiple shoot growth induction, explants exposed to TDZ were transferred to shoot proliferation medium [MS medium containing B5 vitamins supplemented with BAP (1.0 - 10 µM), kinetin (1.0 -10 µM) in combination with NAA at two levels (0.05 and 0.10 µM)]. After 4 to 5 wk, percentage of explants produced multiple shoots and numbers of shoots per explants were recorded. After 4 to 6 wk of induction culture and 4 to 5 wk of shoot proliferation, the explants with regenerated shoots (ht around 1 cm) were transferred onto the shoot elongation medium containing half strength MS salts, B5 vitamins, L-glutamine (400 mg^{-1}) supplemented with GA_3 at different concentrations (0.5 to 5.0 µM). The cultures were maintained 2 wk in above medium without subculturing and then transferred to half-MS medium for elongation of shoots and also to drain the excess hormone to the medium.

Rooting of plantlet

Induction of rooting was found to be an extremely difficult process in Co7 papaya cultivar. Hence, different hormonal combinations were tried to find

a suitable media for rooting. Green and healthy elongated shoots with 2-3 trilobed leaves were excised from mother tissue and cultured on half-strength MS solid medium supplemented with IBA at 0.0, 1.5 2.5, 3.5 5.0 μM for root induction. They were incubated at 16 h photoperiod for 2 to 3 wk. After 3 wk of culturing, the frequency of explants producing roots and the average number of roots per shoot and root length were recorded.

Hardening and acclimatization

In vitro regenerated plantlets were washed carefully in running tap water to remove the traces of agar. They were transferred to culture tubes containing quarter-strength liquid MS salts without sucrose for 24-48 h and then transferred to pots containing autoclaved soil and soilrite (1:1, w/w) and were covered with polybags for 4 wk to maintain high RH. The plantlets were initially irrigated with quarter-strength inorganic salts of MS medium for 2 wk followed by tap water. Potted plantlets were grown in culture room conditions (25±2°C, 55±5% RH, under 16 h of photoperiod with a light intensity of 40 μmol m^{-2} s^{-1}) for 2 months. Polybags were removed gradually upon emergence of new leaves and acclimatized plantlets were transferred to the greenhouse.

Statistical analysis.

The design of all experiments was a completely randomized design. Twenty cultures were raised for each treatment, and all experiments were performed three times. Analysis of variance and Duncan's multiple range tests were used for comparison among treatment means and significance was determined at $P \leq 0.05$ levels.

Results and discussion

Adventitious shoot initiation

About 75% of *Carica papaya* var. Co7 seeds germinated with in 10-15 days of inoculation on a growth regulator free half-strength MS medium. Epicotyl segments excised from the *in vitro* grown seedlings were used as explants in the present study. Shoot bud induction and proliferation varied with the age of explants. A significantly greater percentage response was recorded from 10 days-old explants (data not shown). Adventitious shoot induction in papaya was highly influenced by both of cytokinin and explant type.

Adventitious shoots were not observed in cytokinin-free medium (control treatment). On the other hand, MS basal medium supplemented with various

levels of TDZ showed swelling of explants, followed by differentiation of green shoot initials with different regeneration frequencies. However, the initiation of shoots was slow in all the concentrations of TDZ.

The induction of adventitious shoots was enhanced by increasing the concentration of TDZ from 0.5 to 2.5 µM. The highest percentage of explants (80.50 %) producing shoots (6.3±0.14) was observed with 2.5 µM TDZ (Table 1). This finding is in accordance with an earlier report (De Pavia Neto *et al*., 2003; Liu *et al*., 2003; Ahmad and Anis, 2007).

Table 1. Effect of TDZ on shoot organogenesis in epicotyl explants of Co7 papay cultivar.

TDZ concentration (µM)	percentage of explants responded	Number of shoots per explants
0.0	0.0±0.0 e	0.0±0.0 e
0.5	44.80±1.49 cd	2.1±0.2 d
2.5	80.50±1.85 a	6.3±0.14 a
5.0	60.25±1.11 b	2.9±0.7 ab
7.5	50.13±1.05 c	1.7±0.4 b
10	34.75±2.06 d	1.40±.6 c

Values followed by the same letter are not significantly different at $P<0.05$ according to Duncan's multiple range tests.

TDZ is extensively used for the induction of shoot regeneration in several plant species. A decrease in the number of shoots was noticed when the concentration of TDZ was increased from 2.5 to 10 µM. The lowest percentage of explants (34.75±2.06) producing shoots (1.4±0.6) was observed with highest concentration of TDZ (10.0 µM). Husain *et al*. (2007) have also supported the above findings that frequency of shoot regeneration ability declined markedly at higher concentrations of TDZ and was invariably associated with thick and stunted shoots.

Explants cultured on MS medium supplemented with different concentration of TDZ at different time periods (4, 5 or 6 wk), showed different responses. The frequency of shoot organogenesis, number of shoots/explants was found to vary significantly depending on the concentration of TDZ, as well as different time periods. However, the best concentration of TDZ (2.5 µM) produced (6.3±0.14) shoots was noted after 6 wk of culturing.

Moreover, cultures grown continuously on TDZ supplemented medium after 5 or 6 wk resulted in formation of stunted/suppressed shoots, which leads to death of primary induced shoot initials. These deleterious effects on adventitious shoot induction and proliferation of explants of longer exposure have been already reported (Ahmad and Anis, 2007 and Husain *et al*., 2007).

Shoot proliferation and elongation

The shoot proliferation and elongation was performed with the aim to overcome barriers form TDZ induced cultures i.e. 1) high concentration of TDZ induced adventitious shoots were mostly incomplete plant and difficult to count (Fig 1a). These shoots showed poor elongation as well as rooting when they transferred to half-MS medium without the addition of any growth regulator. 2) TDZ (2.5 µM) induced only (6.3±0.14) shoot initials was noted after 4 wk of culture.

To enhance further shoot proliferation, TDZ only (2.5 µM) induced cultures after 4 wk were transferred to shoot proliferation medium [MS basal medium supplemented with B5 vitamins with varying levels of BAP or kinetin with fixed concentration of NAA (0.05 or 0.01 µM)] (Table 2).

Table 2. Effects of combination of plant growth regulators on shoot multiplication of Co7 papaya cultivar.

Growth regulators used in the treatment		percentage explants producing shoots	Number of shoots per explant
Combination	**Concentration (µM)**		
Control	0.00	22+2.8d	1.0+0.1
BAP+NAA	1.0+0.05	58.4±5.8 b	9.4±1.4 b
	3.0+0.05	66.4±6.2 b	10.2±1.3 b
	5.0+0.05	76.1±6.1a	22.1±2.1 a
	7.0+0.05	68.7±6.3 b	10.7±1.4 b
	10.0+0.05	56.1±5.2 b	6.2±2.2 c
	1.0+0.10	55.3±5.6 b	5.6±2.2 c
	3.0+0.10	52.7±4.6 b	5.1±1.8 c
	5.0+0.10	45.9±4.8 c	3.9±1.7 c
	7.0+0.10	43.3±4.3 c	3.2±2.3 c
	10.0+0.10	38.1±3.3 c	2.0±1.2 c
Kinetin+NAA	1.0+0.05	56.6±3.7 b	6.5±2.2 c
	3.0+0.05	57.3±3.8 b	9.5±1.5 b
	5.0+0.05	58.8±3.9 b	9.6±1.5 b
	7.0+0.05	54.1±4.2 b	5.9±1.9 c
	10.0+0.05	69.5±5.1 a	12.7±2.1a
	1.0+0.10	39.1±3.1 c	2.6±2.2 c
	3.0+0.10	35.9±1.8 c	1.9± 0.2 d
	5.0+0.10	26.2±2.3c	1.6±0.2 d
	7.0+0.10	26.6±2.1c	1.6±0.2 d
	10.0+0.10	25.5±1.3 c	1.3±0.1 d

All explants have been exposed to 2.5 µM for 4 wk before transfer to shoot multiplication medium.Values represent means±SE of 20 replicates per treatment in three repeated experiments. Values followed by the same letter are not significantly different at $P<0.05$ according to Duncan's multiple range tests.

The transfer of TDZ induced cultures to shoot multiplication medium for shoot multiplication was already reported by Ahmad and Anis (2007). Among the two cytokinins tested, BAP+NAA was found to be the best and optimum response in terms of percentage of explants producing shoots and the highest

number of shoot per explant was recorded on proliferation medium supplemented with BAP (5.0 µM) and NAA (0.05 µM). On this medium 76.10 % cultures responded with an average 22 shoots per culture. This observation agreed with the earlier reports of Ahmad and Anis (2007). They suggested that cytokinin: Auxin ratio is an important factor for the differentiation of adventitious shoots as elucidated by Skoog and Miller (1957).

The cultures exposed to BAP (5.0 µM) and NAA (0.05 µM) produced normal shoots with reduced height (Fig.1, b1). These cultures were transferred to shoot elongation medium for elongation of the shoots. Eapen et al. (1998) reported that it was essential to transfer the TDZ induced shoots to medium containing GA3 for elongation. The elongation medium contained half-strength MS medium supplemented with different concentration of GA_3 (0.5, 1.0, 1.5, 2.0, 2.5 µM) and maintained for only 7 days without sub-culturing. They were subsequently transferred to growth regulator free medium for another 3 wk. Maximum (55%) shoot elongation with an average shoot length of 2 to 3 cm was observed at 1.5 µM GA_3 (data not shown), (Fig.1, b2).

In vitro root induction and acclimitization

Induction of rooting was found to be an extremely difficult process in Co7 papaya. Hence, different hormonal combinations were tried to find a suitable media for rooting. Shoots of 1.5 cm ht with 2-3 trilobed leaves were transferred onto a rooting mediun [half-strength MS basal medium,3% sucrose] supplemented with varying levels of IBA and cultured for 2 wk under 16 h photoperiod. Half-strength MS medium without the addition of any growth regulator (control) failed to induce root formation even after 4 wk (Table 3).

Table 3. Effect of IBA on adventitious shoots of Co7 papaya cultivar.

IBA concentration (µM)	Rooting (%)	Number of roots per shoot	Root length (cm)
0.0	0.0±0.0e	0.0±0.0e	0.0±0.0 e
0.5	30.6±2.0c	3.1±0.2b	2.0±0.5 c
1.5	45.0±7.0b	3.4±0.4b	2.0 ±0.6 b
2.5	60.0±6.0a	5.0±0.7 a	3.5±0.5 a
3.5	38.5±6.0c	2.2±0.4c	1.9±0.8 d
5.0	27.0±4.0d	0.9±0.6d	1.3±0.4 d

Values followed by the same letter are not significantly different at $P<0.05$ according to Duncan's multiple range test.

Primary root induction was observed on 12th days onwards (Fig 1c). A maximum frequency of root formation (60%) and the highest number of roots

(5±0.7) with maximum root length (3.5±0.5 cm) was achieved on half-strength MS medium with IBA (2.5.0 µM) after 3th wk (Fig 1d). It was observed that rooting of papaya shoots, along with root length, increased with increasing IBA concentration in the rooting medium. However, it decreased with increasing level of IBA beyond 5.0 µM (Table 3). These short roots allowed plantlets a better acclimatization by avoiding root damage during transplantation to soil. After transplantation, the plantlets recorded normal root elongation, and approximately 72% of transplanted plantlets were successfully acclimatized (Fig. 1e).

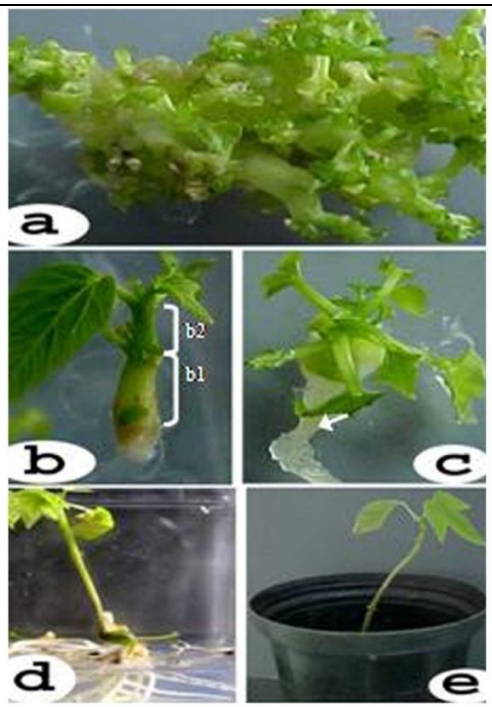

Fig.1. a) Multiple shoot induction on MS medium with BAP (5 µM) ±NAA (0.05 µM)., b) Elongation on half MS with 1.5 µM of GA_3., b1) stunted shoot before GA_3 treatment., b2) elongated shoot after GA_3 treatment., c) Rooting of *in vitro* regenerated shoots half MS with 2.5 µM of IBA., d) Plant with well developed root system ready for hardening., e) Plant acclimatized to greenhouse conditions

Conclusion

The *in vitro* protocol described in this study for regenerating plantlets of *Carica papaya* using epicotyl segments is simple and reproducible. Since the plantlets were developed directly without intervening of callus phase, somoclonal variation among the regenerates is avoided. This protocol could

thus be useful for improvement, conservation, and large-scale planting of this economically important fruit-yielding tree.

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