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Multiple shoot regeneration from immature embryo explants of papaya

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Abstract

A simple and rapid method for multiple shoot formation *in vitro* from immature embryo axis explants of *Carica papaya* L. cvs. Honey Dew, Washington and Co2 is described. Multiple shoot regeneration was achieved by culture of the explants on modified Murashige and Skoog (MS) medium supplemented either with thidiazuron (TDZ; 0.45-22.7 μM) or a combination of benzylaminopurine (BAP; 0.2 - 8.84 μM) and naphthalene acetic acid (NAA; 0.5 - 2.64 μM). Highest frequency of shoot regeneration occurred on medium supplemented either with 2.25 μM TDZ or a combination of BAP (4.4 μM) and NAA (0.5 μM). Composition of the basal media influenced the frequency of multiple shoot initiation. Stunted shoots regenerated at 4.5 μM and higher concentrations of TDZ. Such shoots could, however, be elongated by transfer to medium containing 5.7 μM GA_3. Rooting of the regenerated shoots was achieved in presence of indolebutyric acid (IBA; 4.92 - 19.68 μM), however, least response was in presence of 14.7 μM IBA. Rooted plants were hardened and transferred to pots.

Additional key words: BAP, *Carica papaya*, IBA, NAA, thidiazuron.

Introduction

In vitro regeneration of *Carica papaya* L. has been achieved *via* organogenesis (Rajeevan and Pandey 1986, Winner 1988, Reuveni *et al.* 1990, Hossain *et al.* 1993, Bhattacharya and Khuspe 2001) and somatic embryogenesis (Chen *et al.* 1987, Fitch 1990, Fitch and Manshardt 1993, Bhattacharya *et al.* 2002).

Thidiazuron (TDZ), a substituted phenyl urea used as a defoliant (Yip and Yang 1986), also exhibits cytokinin like activity (Magioli *et al.* 1998). It has been used to induce adventitious shoots in a number of plant species (Sujatha and Reddy 1998, Eva 1999). In papaya tissue cultures, combinations of BAP and NAA are used for multiple shoot induction (Rajeevan and Pandey 1986, Hossain *et al.* 1993). However, TDZ has not been tried for *in vitro* shoot formation in this plant species. The present communication reports on the effect of TDZ on multiple shoot formation from the immature embryo axis explants of three cultivars of papaya and compares this with the response elicited by the use of the most commonly used hormonal combination of BAP and NAA.

Materials and methods

Out of the three papaya (*Carica papaya* L.) cultivars, Honey Dew is monoecious, Co2 and Washington are dioecious. The plants were grown in greenhouse. Immature fruits were collected from the selfed cv. Honey Dew and the sib mated cvs. Co2 and Washington, 90 - 115 d post anthesis. The fruits were surface sterilized (Bhattacharya and Khuspe 2001), cut transversely under aseptic conditions and the immature seeds collected in a sterile Petri dish. Immature zygotic embryos were excised from the seeds and used as the explants. MS (Murashige and Skoog 1962), B_5 (Gamborg *et al.* 1968), White's (1963) and a combination of MS salts, B_5 vitamins and 26.6 μM glycine (designated as MBG medium) were used as the basal nutrient media in the present study. Sucrose, fructose, glucose or maltose, each at 30 g dm^{-3}, were used individually as the carbon sources. Different

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Abbreviations: BAP - benzylaminopurine; B5 - Gamborg medium; IBA - indolebutyric acid; MS - Murashige and Skoog; NAA - naphthalene acetic acid; TDZ - thidiazuron.

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concentrations of TDZ (0.45 - 22.7 μM) and a combination of BAP (0.2 - 8.87 μM) and NAA (0.5 - 2.64 μM) were used to further supplement the media. The medium pH was adjusted to 5.8, solidified with 0.7 % agar and autoclaved at 1.5 kg cm^{-2} for 20 min. Medium (20 cm^3) was poured into each pre-sterilized Petri dish and allowed to solidify. The cultures were incubated at 25 ± 2 °C under continuous cool white fluorescent light (irradiance of 25 μmol m^{-2} s^{-1}). Subculture was carried out at 2 week intervals. The explants were also cultured on phytohormone free media, which served as the control. Shoot elongation was achieved in media containing 5.8 μM GA_3. MBG basal medium supplied with IBA (4.9 - 19.7 μM) was used for rooting the regenerated shoots. Twenty explants in replicates of three were analyzed in each experiment. All experiments were repeated three times. Data obtained were subjected to *ANOVA* and Student's *t*-test and least significant difference was calculated at 5 % level of confidence.

Results and discussion

The immature embryo axis explants showed visible swelling within a week of culture initiation (Fig. 1*A*). On the shoot multiplication media, the meristematic region of the embryo axis formed white watery callus at its base. In the second week of culture, green shoot initials were seen on the apical region of the embryo axis. These initials later developed into shoots in about six weeks. In all the papaya cultivars tested, maximum average number of shoots were regenerated per explant in MBG medium supplemented with 2.2 μM TDZ (Fig. 1*B*). While no multiple shoots were regenerated on basal MBG medium, the number of regenerants increased with the increasing concentration of TDZ from 0.45 to 2.2 μM in the medium. Best response was achieved at 2.2 μM TDZ. At this concentration the average number of shoots regenerated were 14.62 ± 1.88 for cv. Honey Dew, 14.51 ± 2.06 for Co2 and 14.57 ± 1.9 for cv. Washington, respectively (Table 1). TDZ concentrations of 4.5 μM in the medium resulted in stunting and fasciation of the shoots. With further increase of TDZ concentrations the phenomenon became progressively acute to the extent that at 9.0 μM TDZ concentration, the number of the regenerated shoots could not be counted (Table 1). The multiple shoot regeneration response in presence of TDZ was closely followed by response in presence of BAP + NAA. In BAP + NAA containing medium the number of shoots regenerated from the embryo axis increased with increasing concentrations of the phytohormones. Maximum number of shoots regenerated (14.27, 14.17 and 14.44 for cultivars Honey Dew, Co2 and Washington) was in presence of 4.44 μM BAP and 0.54 μM NAA (Fig. 1*D*, Table 1). Further increase in the concentration of the phytohormones resulted in progressive decrease in the number of shoots formed. This, however, did not rule out the possibility of the presence of shoot meristems in the cultured tissues. The

Table 1. Effect of TDZ and BAP + NAA on multiple shoot formation in papaya cultivars Honey Dew (HD), Co2, and Washington (WA) on MBG basal nutrient medium (n.d. - unable to count shoots). Means ± SE, $n = 20$.

Regulators	Conc. [μM]	Explants forming multiple shoots [%]			Number of shoots [explant^{-1}]		
		HD	Co2	WA	HD	Co2	WA
Control	0.00	-	-	-	1.00	1.00	1.00
TDZ	0.45	62.20 ± 0.58	60.00 ± 1.00	63.33 ± 1.00	4.22 ± 1.35	4.66 ± 1.50	4.53 ± 1.50
	0.91	86.66 ± 1.00	80.00 ± 1.00	85.53 ± 1.52	5.59 ± 1.57	5.55 ± 1.58	5.84 ± 1.73
	2.20	95.53 ± 1.15	91.10 ± 0.58	94.43 ± 1.52	14.62 ± 1.88	14.51 ± 2.06	14.57 ± 1.90
	4.50	71.10 ± 1.15	66.66 ± 2.00	67.76 ± 2.88	7.64 ± 1.54	7.91 ± 1.68	7.84 ± 1.67
	6.80	44.43 ± 0.58	46.66 ± 1.73	47.76 ± 1.52	4.89 ± 1.80	5.11 ± 2.12	5.14 ± 2.01
	9.00	44.43 ± 1.52	48.86 ± 1.53	46.66 ± 1.00	n.d.	n.d.	n.d.
BAP+NAA	0.22 + 0.54	8.86 ± 0.58	10.00 ± 1.00	10.00 ± 0.38	2.77 ± 0.38	2.89 ± 0.38	3.05 ± 0.38
	0.22 + 2.64	20.00 ± 2.00	18.43 ± 1.52	18.87 ± 0.80	3.43 ± 0.80	3.37 ± 0.80	3.66 ± 0.80
	0.44 + 0.54	48.90 ± 0.58	47.77 ± 0.58	46.67 ± 1.36	4.40 ± 1.36	4.18 ± 1.36	4.27 ± 1.36
	0.44 + 2.69	80.00 ± 1.00	82.20 ± 0.58	81.10 ± 1.71	6.22 ± 1.71	6.02 ± 1.71	6.17 ± 1.71
	2.22 + 0.54	76.66 ± 1.00	76.67 ± 1.00	77.77 ± 1.53	7.26 ± 1.53	7.09 ± 1.54	7.15 ± 1.54
	2.22 + 2.69	85.53 ± 0.58	84.43 ± 0.58	83.33 ± 2.54	8.80 ± 2.54	8.83 ± 2.54	8.98 ± 2.54
	4.44 + 0.54	92.20 ± 0.58	91.10 ± 0.58	93.33 ± 3.93	14.27 ± 3.93	14.17 ± 3.93	14.44 ± 3.93
	4.44 + 2.69	85.33 ± 0.58	82.20 ± 1.15	83.33 ± 2.79	10.11 ± 2.79	9.93 ± 2.79	10.08 ± 2.80
	8.87 + 0.54	23.33 ± 1.00	22.23 ± 1.15	21.10 ± 1.42	4.03 ± 1.42	3.47 ± 1.41	3.92 ± 1.42
	8.87 + 2.69	11.10 ± 1.53	12.23 ± 1.15	10.00 ± 1.15	3.42 ± 1.15	3.00 ± 0.00	3.00 ± 0.00

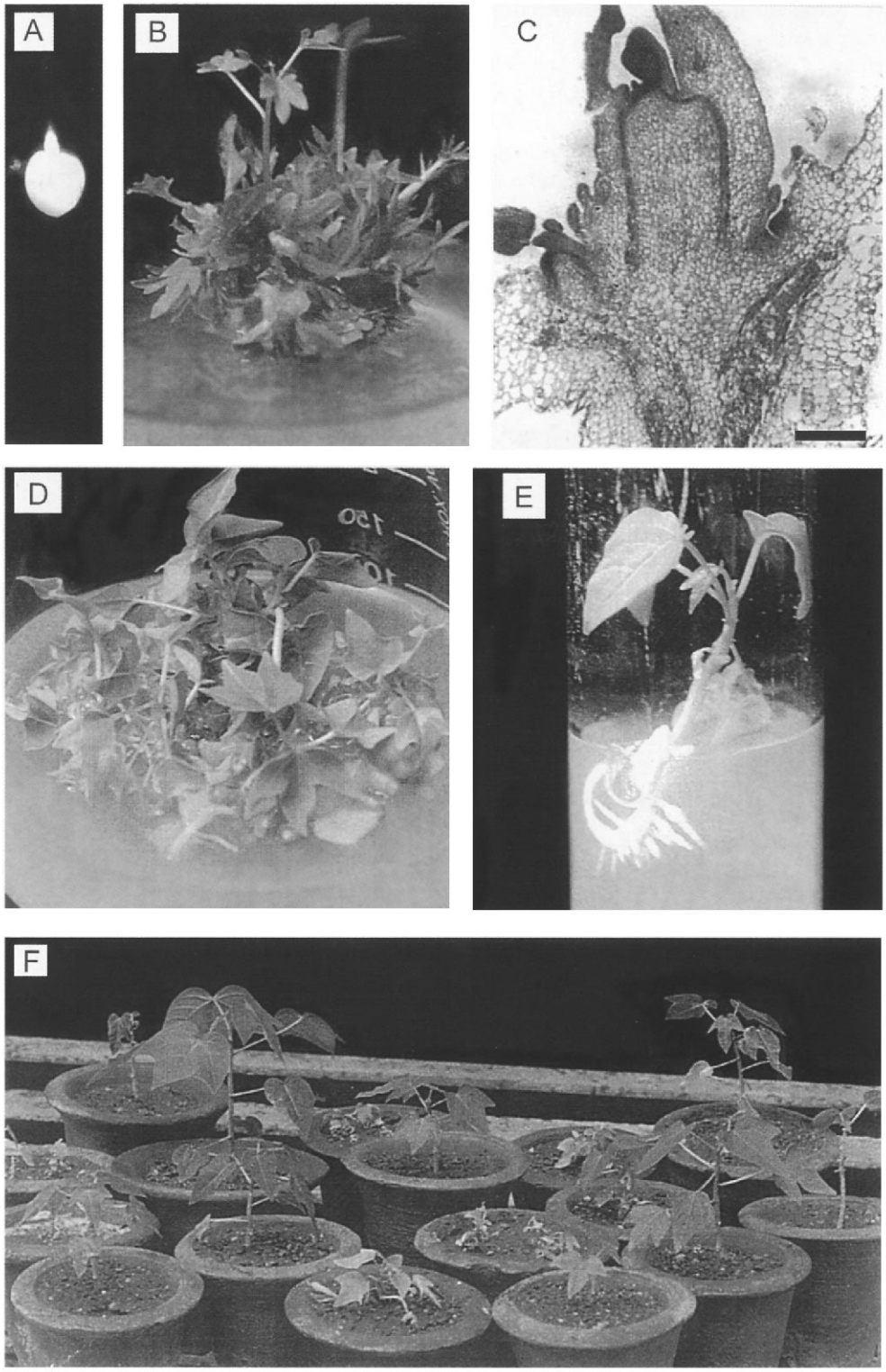

Fig. 1. Stages of multiple shoot formation from immature embryo axis of papaya. *A* - Immature embryo axis used as explant; *B* - multiple shoots formed in TDZ media; *C* - transverse section of an explant shows multiple shoot formation, *bar* = 250 μm; *D* - multiple shoots formed in BAP + NAA medium; *E* - single shoot showed root formation; *F* - multiple shoots regenerated plant in soil.

development of these meristems may have been inhibited by larger shoots. Although multiple shoot regeneration was observed over a wide range of TDZ concentration and a combination of BAP + NAA in the medium, incorporation of 2.2 μM TDZ or a combination of 4.4 μM BAP + 0.5 μM NAA supported the most prolific adventitious shoot regeneration (Table 1). Histological examination revealed the origin of the shoots from the apical region of embryo axis (Fig. 1*C*).

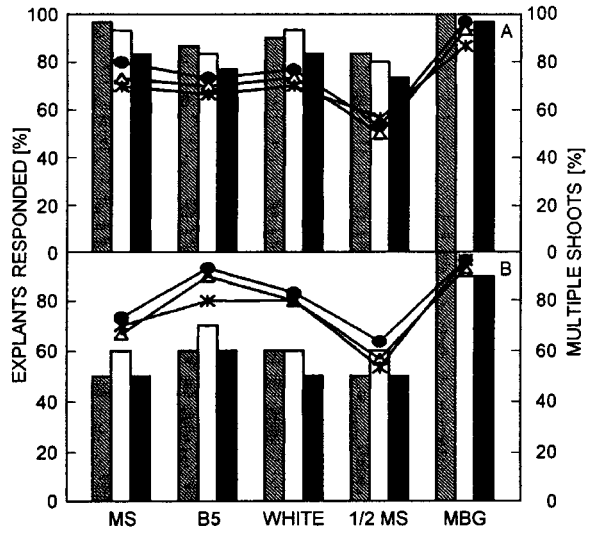

Fig. 2. Effect of different basal media using 4.4 μM BAP + 0.5 μM NAA (*A*) or 2.2 μM TDZ (*B*) on percentage of responded explants (*columns*) and multiple shoot formation (*lines*). HD - *stripped columns, circles*; Co2 - *empty columns, triangles*; WASH - *full columns, asterisks*.

Different basal media, *viz.* MS, B_5, White's, 1/2 MS and MBG were supplemented with 2.2 μM TDZ or a combination of BAP (4.4 μM) and NAA (0.5 μM), and evaluated for their influence on multiple shoot regeneration from immature embryo axis explants of papaya. MBG medium was found to be the best basal medium as far as the initial swelling and callusing of the explant was concerned. Ninety six percent of the explants cultured on this medium responded (Fig. 2). Lowest response was obtained on ½ MS basal medium. Regeneration of maximum multiple shoots per explant was also achieved with the use of MBG basal medium supplemented either with 2.2 μM TDZ (14.03 ± 1.3 in Co2, 14.2 ± 1.46 in Honey Dew, 14.13 ± 1.15 in Washington) or a combination of BAP (4.4 μM) and NAA (0.5 μM), (14.73 ± 1.2 in Co2, 14.06 ± 1.3 in Honey Dew, and 14.43 ± 1.19 in Washington).

To affect elongation of the regenerated shoots, these along with the mother explant were shifted to the MS basal medium without any phytohormone. The stunted shoots regenerated in presence of higher concentration of TDZ (4.5 μM and above) were transferred to GA_3 (5.7 μM) containing MS basal medium for 15 d for shoot elongation. The phenomenon of stunting and fascination of shoot was not observed with the incorporation of BAP and NAA in the regeneration media.

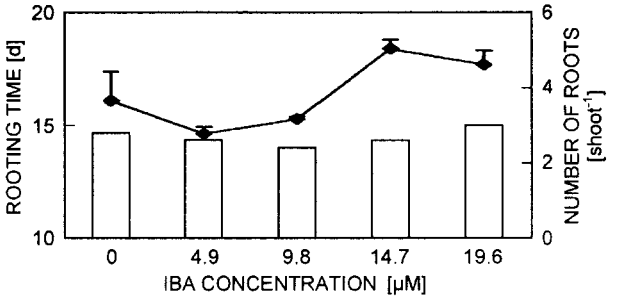

Fig. 3. Effect of IBA concentrations incorporated in MBG basal medium (for all the three cultivars), on root formation from *in vitro* regenerated shoots of papaya (rooting time - *columns*, number of roots - *rhombs*).

Well developed and elongated shoots (3 - 5 cm in height) were excised after 15 - 20 d and transferred to half strength MBG medium supplemented with 4.9 - 19.6 μM IBA and 3 % sucrose. The shoots developed roots after 2 weeks of incubation in all the media tested. In all the three cultivars maximum number of roots per shoot were induced in media containing 14.7 μM IBA (Fig. 2). Similar IBA effect on papaya root formation was achieved earlier (Mondal *et al.* 1990). Phytohormone free basal medium served as the control where 3.16 ± 0.76 roots per shoot were produced. Lowest rooting response was observed on medium containing 4.9 μM IBA (Fig. 3). The rooted shoots (Fig. 1*E*) were transferred to pots containing a mixture of sand:soil:compost (1:1:1) (Fig. 1*F*) and hardened. About 76 % of the plants survived under greenhouse condition and these were later planted outside.

The present communication describes a rapid and efficient protocol for the development of rooted and hardened plants from three cultivars of papaya. Compared with earlier reports on multiple shoot formation in papaya (Rajeevan and Pandey 1986, Winner 1988, Mondal *et al.* 1990, Hossain *et al.* 1993) the present process does not require different media for establishment of cultures, and for proliferation and further development of the shoots. The procedure has the added advantage of using immature zygotic embryo as the explant source, which is aseptic and hence the chance of contamination is minimized. Embryo axis is now being preferred for transformation experiments (Krishnamurthy *et al.* 2000, Polowick *et al.* 2000) due to its smaller size, which favors handling of a large number of explants at one time and also it takes less time to develop shoots.

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