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RESEARCH ARTICLE

Effect of nutritional and growth hormonal factors on *in vitro* regeneration of papaya (*Carica papaya* L. cv. Red Lady)

Van-Hong Nguyen [1], Chung-Ruey Yen[2] and Ching-Hsiang Hsieh[2*]

[1] *Department of Tropical Agriculture and International Cooperation, National Pingtung University of Science and Technology, Pingtung 91201, Taiwan (R.O.C).*

[2] *Department of Plant Industry, National Pingtung University of Science and Technology, Pingtung 91201, Taiwan (R.O.C).*

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Abstract: An efficient *in vitro* regeneration system was developed for the hermaphrodite papaya 'Red Lady' using nodal explants. Nodal explants were cultured on modified Murashige and Skoog (MS) medium supplemented with 3 6-benzylaminopurine (BAP) concentrations (0.0, 0.5 and 1.0 mgL^{-1}) or kinetin (10 mgL^{-1}) in combination with 1-naphthaleneacetic acid (NAA) (0.0, 0.1, 0.3, 0.4 and 0.5 mgL^{-1}). Media consisted of 0.5 or 1.0 mgL^{-1} BAP in combination with 0.1 mgL^{-1} NAA were determined to be the most effective based on the percentage of explants that produced shoots (93.33 and 86.67 % , respectively), mean number of shoots (1.79 and 1.81 shoots, respectively) and shoot height (2.26 and 1.95 mm, respectively). Shoot multiplication and elongation was achieved 30 days after transferring the initiated shoots to full-strength MS supplemented with 0.5 mgL^{-1} BAP, 0.1 mgL^{-1} NAA, and 200 – 400 mgL^{-1} casein hydrolysate (CH). Effect of full and haft-strength MS medium supplemented with different indole-3-butyric acid (IBA) concentrations (0.0 – 4.0 mgL^{-1}) was tested for rooting. Adventitious rooting occurred after subculturing excised shoots on a medium containing 2.0 or 2.5 mgL^{-1} IBA and 300 mgL^{-1} CH, and plantlets were successfully acclimatised on potting medium consisting of peat moss: perlite (1:1). After 40 days of acclimatisation, plantlets grew normally and vigorously. This protocol can be used for commercial production of 'Red Lady' papaya plantlets.

Keywords: 1-naphthaleneacetic acid, 6-benzylaminopurine, casein hydrolysate, indole-3-butyric acid, MS medium.

INTRODUCTION

Papaya (*Carica papaya* L.) is a polygamous species with three forms of inflorescences: male, female and hermaphrodite (Paull & Duarte, 2011). It is one of the few fruit crops still propagated mainly by seeds. Inherent heterozygosity, production of non-true-to-types and susceptibility to papaya ring spot virus are some problems which hindered the propagation of papaya by seeds (Teixeira da Silva *et al.*, 2007; Clarindo *et al.*, 2008). It was also reported that undesirable male plants, which are useless, prevail as high as 30 % and sometimes over 50 % of trees planted from seeds in papaya fields (Jordan *et al.*, 1983). In addition, plants grown from seeds show considerable variation in disease susceptibility, fruit quality and yield (Teixeira da Silva *et al.*, 2007).

The possibility of developing planting materials, which are highly productive and resistant to diseases with similar characteristics of the papaya mother plants through asexual propagation has been reported (San Jose & Marim, 1988). Conventional asexual propagation techniques, such as rooting of cutting and grafting have been successful (Ramkhelawan & Baksh, 1998; Chong *et al.*, 2008). However, they are often tedious and impractical to be carried out on large scale due to the limited number of plants produced by mother plant (Setargie *et al.*, 2015). In addition, these methods cannot be used to exclude transmission of systemic diseases from mother plants. Micropropagation represents an economic way of continuously producing new uniform true-to-parental type planting materials of known superior lines (Roy *et al.*, 2012; Mumo *et al.*, 2013).

* Corresponding author (hsieh@mail.npust.edu.tw; https://orcid.org/0000-0002-7764-6497)

In vitro propagation techniques can be employed to produce a large number of true-to-type high-quality planting material in papaya cultivation. Furthermore, the techniques can be used as an important tool in crop improvement programmes since they help to overcome problems experienced in conventional breeding methods and lead to rapid clonal production of crops.

Several studies on *in vitro* propagation of different papaya cultivars using explants with seedling origin (Ascencio-Cabral *et al.*, 2008; Farzana *et al.*, 2008) as well as from asexual organs (Winnaar, 1988; Kabir *et al.*, 2007; Panjaitan *et al.*, 2007; Teixeira da Silva *et al.*, 2007; Wu *et al.*, 2012; Ambasta & Kumari, 2013; Setargie *et al.*, 2015) have been reported. However, the stages of *in vitro* propagation of papaya respond differently to variation of explants, genotype of explants as well as nutritional and hormonal composition of the medium (Winnaar, 1988; Usman *et al.*, 2002; Mumo *et al.*, 2013). Therefore, suitable protocols have to be established for different papaya varieties and relevant explants used. 'Red Lady', a hybrid papaya variety, is one of the newly imported cultivars growing in Taiwan and propagated using seeds. Only a small percentage of plantlets used are derived from grafting and cutting methods. Application of *in vitro* propagation technique to produce 'Red Lady' plantlets from nodal segments has not been reported and applied in practice. The present study was conducted to determine the effects of growth regulators, casein and medium composition on *in vitro* propagation of 'Red Lady' papaya from nodal segments.

METHODOLOGY

Plant materials and sterilisation procedure

Newly developed shoot sprouts of 5 – 10 cm were collected from a hermaphrodite 'Red Lady' papaya cultivar growing in a greenhouse. After removing the leaves explants were washed under running tap water for 1 h and taken into the laboratory. Subsequently, lateral buds were sterilised by immersing in 70 % ethanol for 30 s followed by treating with 20 % clolox solution containing 10 drops L^{-1} of tween-20 (sigma, USA) for 15 min. Finally, explants were rinsed 5 times with sterile distilled water, damaged parts removed and separated into single nodal segments (6 mm long) before culturing on MS medium containing 30 gL^{-1} sucrose and 0.65 % agar (w/v). Twenty days after culture establishment uncontaminated explants were transferred on to shoot induction medium.

Shoot induction

Uncontaminated explants were cultured on MS medium (Murashige & Skoog, 1962) supplemented with 6.5 gL^{-1} agar, 30 gL^{-1} sucrose and 6-benzylaminopurine (BAP: 0, 0.5, 1.0 mgL^{-1}), or kinetin (10 mgL^{-1}), alone or in combination with 1-naphthaleneacetic acid (NAA: 0.1, 0.2, 0.3, 0.4, or 0.5 mgL^{-1}). The experiment was replicated 5 times. Each treatment consisted of 12 explants per replicate.

Multiplication and elongation of shoot

Regenerated shoots from 40-day old cultures of MS + BAP (1.0 mgL^{-1}) + NAA (0.1 mgL^{-1}) at the shoot induction step were trimmed to shoots that were 0.5 cm long with 3 leaves, and were subcultured on to MS basal media supplemented with 6.5 gL^{-1} agar, 30 gL^{-1} sucrose, and BAP only (0.0, 0.5 and 1.0 mgL^{-1}) or BAP (0.5 and 1.0 mgL^{-1}) in combination with NAA (0.1, 0.2, 0.3, 0.4 and 0.5 mgL^{-1}) for shoot multiplication and elongation.

Small (0.5 cm, 3 leaves) individual shoots from 40-day cultures of MS + BAP (0.5 mgL^{-1}) + NAA (0.1 mgL^{-1}) were excised carefully and transferred to a medium containing MS basal medium, agar (6.5 gL^{-1}), sucrose (30 gL^{-1}), BAP (0.5 mgL^{-1}), NAA (0.2 mgL^{-1}) (Sign: MSC), and different concentrations of casein hydrolysate (CH) (Merck, Germany) (0, 50, 100, 200, 300, 400, and 500 mgL^{-1}) to determine the effects of CH on elongation and multiplication of shoot. The experiment was replicated 5 times. Each treatment consisted of 6 explants per replicate.

Rooting of shoot

Shoots (length of 1.5 cm) from 30-day old cultures in MSC medium with CH (300 mgL^{-1}) were separated individually and transferred to rooting media containing full and half-strength MS salts supplemented with agar (6.5 gL^{-1}), sucrose (30 gL^{-1}), CH (300 mgL^{-1}), and different concentrations of indole-3-butyric acid (IBA: 0.0, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, or 4.0 mgL^{-1}). The experiment was replicated 5 times. Each treatment consisted of 6 explants per replicate.

Culture conditions and statistical analysis

Cultures were incubated at 25 ± 2 ºC with 16 h/8 h of day/night under a cool white fluorescent light delivering approximately 45 µmol m^{-2} s^{-1}. The research was carried out in the Exploration and Development of Potential

Ornamental Plant Material Laboratory, Department of Plant Industry, National Pingtung University of Science and Technology, Taiwan from February 2015 to February 2017.

All experiments were arranged as completely randomised designs. Data were analysed using one-way ANOVA for shoot initiation, shoot proliferation and acclimatisation or two-way ANOVA for shoot rooting, and when F-test showed significant treatment results, separation of treatment means were determined using Duncan's multiple range test (DMRT) at $p < 0.01$.

RESULTS AND DISCUSSION

Shoot induction

In this study, nodal explants were responsive to all treatments tested with or without plant growth regulators (PGR) indicating the high regenerative potential of nodal explants. Shoot regeneration began at week 3 of culture with visible proliferation of buds from the nodes (Figure1 A, B). Results of *in vitro* shoot regeneration from single node explants exposed to different PGR concentrations after 40 days of culture are shown in Table 1. MS media devoid of PGR or high NAA concentration in combination with BAP showed the lowest response of shoot initiation. Shoot regeneration from node explants cultured on MS

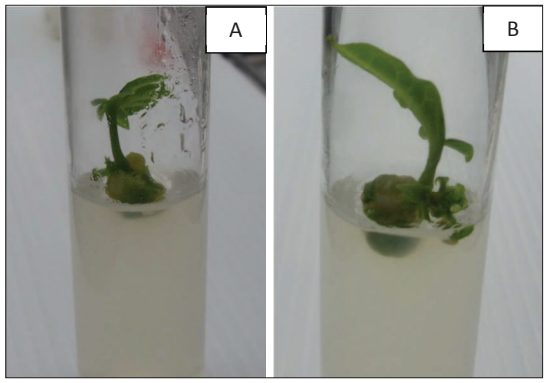

Figure 1: Shoot induction from nodal explants of papaya. (A) shoot regeneration began by week 3 of culture; (B) proliferation of shoot from the node of the explants in MS + BAP (1.0 mgL^{-1}) + NAA (0.1 mgL^{-1}).

Table 1: Effect of BAP, kinetin in combination with NAA in MS medium on shoot induction from nodal explants of papaya (after 40 days of culture)

Growth regulators (mgL^{-1})			Percentage of explants produced shoots (mean ± SE)	Mean number of shoots (mean ± SE)	Shoot height (mm) (mean ± SE)
BAP	Kinetin	NAA			
0.0		0.0	6.67 ± 3.73 h	0.40 ± 0.55 d	0.70 ± 0.97 hi
0.5		0.0	43.33 ± 3.73 ef	1.00 ± 0.00 c	2.11 ± 0.67 abc
1.0		0.0	53.33 ± 4.56 de	1.00 ± 0.00 c	2.14 ± 0.25 ab
0.5		0.1	93.33 ± 3.73 a	1.79 ± 0.16 a	2.24 ± 0.28 a
1.0		0.1	86.67 ± 4.56 a	1.81 ± 0.18 a	1.95 ± 0.08 abcde
0.5		0.2	60.00 ± 3.73 cd	1.30 ± 0.24 bc	1.76 ± 0.06 abcdefg
1.0		0.2	70.00 ± 4.56 bc	1.34 ± 0.15 bc	1.67 ± 0.12 abcdefg
0.5		0.3	36.67 ± 4.56 f	1.00 ± 0.00 c	1.43 ± 0.15 efg
1.0		0.3	43.33 ± 6.97 ef	1.17 ± 0.24 bc	1.48 ± 0.14 defg
0.5		0.4	23.33 ± 3.73 g	1.00 ± 0.00 c	1.25 ± 0.19 fgh
1.0		0.4	23.33 ± 6.97 g	1.00 ± 0.00 c	1.20 ± 0.19 gh
0.5		0.5	3.33 ± 4.56 h	0.20 ± 0.45 d	0.20 ± 0.45 i
1.0		0.5	6.67 ± 3.73 h	0.40 ± 0.55 d	0.44 ± 0.61 i
	10	0.0	23.33 ± 9.13 g	1.00 ± 0.00 c	1.52 ± 0.33 cdefg
	10	0.1	53.33 ± 7.45 de	1.52 ± 0.21 ab	2.04 ± 0.10 abcd
	10	0.2	73.33 ± 9.13 b	1.31 ± 0.08 bc	1.82 ± 0.12 abcdef
	10	0.3	43.33 ± 9.13 ef	1.30 ± 0.30 bc	1.65 ± 0.19 abcdefg
	10	0.4	40.00 ± 9.13 f	1.07 ± 0.15 c	1.55 ± 0.06 bcdefg
	10	0.5	36.67 ± 7.45 f	1.00 ± 0.00 c	1.50 ± 0.20 defg

Means followed by the same alphabet are not significantly different based on DMRT (at $p < 0.01$)

media supplemented with different combinations of BA (0.5 or 1.0 mgL^{-1}) and NAA (0.1, 0.2, 0.3, 0.4 or 0.5 mgL^{-1}) was higher compared to media supplemented with BAP (0.5, 1.0 mgL^{-1}) alone or 10 mgL^{-1} kinetin in combination with NAA (0, 0.1, 0.2, 0.3, 0.4, 0.5 mgL^{-1}) (Table 1). MS media supplemented with 0.5 or 1.0 mgL^{-1} BAP with 0.1 mgL^{-1} NAA were the most effective media for regeneration of shoots (93.33 and 86.67 %, respectively). Similarly, nodal explants cultured on 0.5 or 1.0 mgL^{-1} BAP in combination with 0.1 mgL^{-1} NAA produced the highest mean number of shoots (1.79 and 1.81 shoots per node, respectively) with highest shoot elongation (2.26 and 1.95 mm, respectively) compared to all the other media tested (Table 1).

In this study, the response of shoot regeneration to combinations of cytokinins (BAP, kinetin) and auxins (NAA) was better compared to the effects of cytokinins (BAP or kinetin) only. This is an evidence for the beneficial effects of the combination of cytokinin and auxin on shoot regeneration as reported earlier for other plants (Suksa-Ard *et al.*, 1997; Sultana & Bari Miah, 2003) as well as for papaya (Panjaitan *et al.*, 2007; Rohman *et al.*, 2007; Roy *et al.*, 2012; Setargie *et al.*, 2015).

Among the two cytokinins (BAP, Kinetin) tested, BAP in combination with NAA was found to be the best in terms of percentage of explants that produced shoots, mean number of shoots and shoot height, and the medium supplemented with BAP (0.5 or 1.0 mgL^{-1}) and NAA (0.1 mgL^{-1}) was found as the best. The results of the present study agrees with Roy *et al.* (2012) who reported higher shoot regeneration from lateral buds on media supplemented with BAP (0.5 mgL^{-1}) in combination with NAA (0.1 mgL^{-1}) compared to BAP or kinetin alone or combinations of kinetin and NAA. Similarly, Winnaar (1988) and Reuveni *et al.* (1990) reported that MS with 0.5 mgL^{-1} BA and 0.1 mgL^{-1} NAA was optimum for regeneration of papaya from lateral bud explants. In contrast, Setargie *et al.* (2015) reported that combination of BAP (1.0 mg L^{-1}) and NAA (0.5 mg L^{-1}) as the best for regeneration while Rohman *et al.* (2007) stated it as BAP (0.5 mgL^{-1}) and NAA (0.20 mgL^{-1}). Thus, the differences observed may be the related response of the genotypes of the plant material used to PGR (Jabeen *et al.*, 2005).

Shoot multiplication and elongation

Effect of PGR combination on shoot multiplication and elongation

Supplementing PGR into MS media was advantageous and promoted both shoot multiplication and elongation (Table 2). MS medium without PGR resulted in poor rate of multiplication, shoot length and number of leaves per shoot (1.09 folds, 5.73 mm and 2.99 leaves, respectively). Incorporation of BAP alone or combination of BAP and

Table 2: Effect of different concentrations of BAP, or combination of BAP and NAA on multiplication and elongation of papaya shoot (after 30 days of culture)

Growth regulators (mgL^{-1})		Multiplication rate (folds) ± SE	Mean shoot length (mm) ± SE	Mean no. of leaves per shoot (leaves) ± SE
BAP	NAA			
0.0	0.0	1.09 ± 0.04 k	5.73 ± 0.08 f	2.99 ± 0.05 h
0.5	0.0	3.52 ± 0.04 e	5.69 ± 0.09 f	3.35 ± 0.06 f
1.0	0.0	4.37 ± 0.05 d	5.22 ± 0.05 g	3.20 ± 0.03 g
0.5	0.1	5.00 ± 0.04 a	6.85 ± 0.05 a	3.90 ± 0.03 a
0.5	0.2	4.53 ± 0.05 c	6.86 ± 0.04 a	3.92 ± 0.03 a
0.5	0.3	2.86 ± 0.05 g	6.43 ± 0.07 bc	3.76 ± 0.04 c
0.5	0.4	1.99 ± 0.03 i	6.22 ± 0.06 de	3.66 ± 0.04 d
0.5	0.5	1.31 ± 0.04 j	5.70 ± 0.12 f	3.56 ± 0.04 e
1.0	0.1	4.71 ± 0.03 b	5.77 ± 0.07 f	3.59 ± 0.04 e
1.0	0.2	4.75 ± 0.03 b	6.50 ± 0.04 b	3.82 ± 0.02 b
1.0	0.3	3.34 ± 0.04 f	6.34 ± 0.06 cd	3.71 ± 0.04 cd
1.0	0.4	2.83 ± 0.07 g	6.12 ± 0.06 e	3.71 ± 0.04 cd
1.0	0.5	2.07 ± 0.02 h	5.79 ± 0.08 f	3.57 ± 0.05 e

Means followed by the same alphabet are not significantly different based on DMRT (at $p < 0.01$)

NAA into MS medium increased shoot multiplication (from 1.31 to 5.00 folds), shoot length (from 5.22 to 6.86 mm) and number of leaves per shoot (from 3.20 to 3.92 leaves).

MS medium supplemented with BAP (0.5 mgL^{-1}) and NAA (0.1 mgL^{-1}) was found to be the most effective combination of growth regulators for shoot formation and shoot multiplication (5.00 folds). In addition, best results for shoot length and number of nodes per shoot were achieved in media consisting of combinations of BAP (0.5 mgL^{-1}) + NAA (0.1 mgL^{-1}) or BAP (0.5 mgL^{-1}) + NAA (0.2 mgL^{-1}). Therefore, MS medium supplemented with BAP (0.5 mgL^{-1}) and NAA (0.1 mgL^{-1}) can be considered as best for elongation and multiplication of shoots.

The ratio of cytokinin and auxin is an important factor for the differentiation of adventitious shoots (Skoog & Miller, 1957). Furthermore, supply of auxin (NAA) at low concentration to the media containing cytokinin (BAP) enhanced shoot proliferation (Reuveni *et al*., 1990). Therefore, the combination of BAP and NAA was used at different rates in shoot multiplication of papaya in many previous studies (Anandan *et al.*, 2011; Ambasta & Kumari, 2013; Mumo *et al.*, 2013). In agreement with these results, use of low NAA concentration (0.1 mgL^{-1}) combined with BAP produced better results compared to higher NAA concentration, and the best results of all parameters were achieved when 0.5 mgL^{-1} BAP and 0.1 mgL^{-1} NAA was incorporated into MS medium. Similar results were reported by Mumo *et al.* (2013). In contrast, Kabir *et al.* (2007) achieved the best result of shoot proliferation at higher NAA (0.5 mgL^{-1}) concentration in combination with BAP (1.0 mgL^{-1}) for papaya variety 'Shahi' showing the interactive effects of genotype and PGR on shoot multiplication.

Effect of casein hydrolysate concentration on elongation and multiplication of shoot

Although MS + BAP (0.5 mgL^{-1}) + NAA (0.1 mgL^{-1}) proved very good for healthy shoot proliferation, shoot elongation was not very satisfactory and the maximum shoot length was 6.86 mm with 3.90 leaves per shoot. To facilitate further shoot multiplication, shoot elongation and increased number of leaves per shoot, individual shoots were transferred to MSC with different concentrations of CH and the results are shown in Figures 2 and 3.

Data indicated that incorporation of CH to MS medium increased shoot elongation as well as shoot multiplication after 30 days of culture (Figure 2) ($p < 0.01$). Incorporation of 200 to 500 mgL^{-1} of CH increased the shoot multiplication rate significantly in the medium containing CH compared to the medium without CH. Although shoot multiplication was achieved in MSC with CH (50 mgL^{-1}), the results were not significantly different from MSC without CH.

Furthermore, quality of shoots, shoot length and number of leaves per shoot in all treatments supplemented with CH was higher than in the treatment without CH at 30 days of culture (Figure 3). Shoot length increased with the increase of CH up to 200 mgL^{-1} and thereafter no significant increase in shoot length was observed (Figure 3). A reduction in shoot length was found in both cases when using 500 mgL^{-1} CH and reducing the CH concentration to lower than 200 mgL^{-1}.

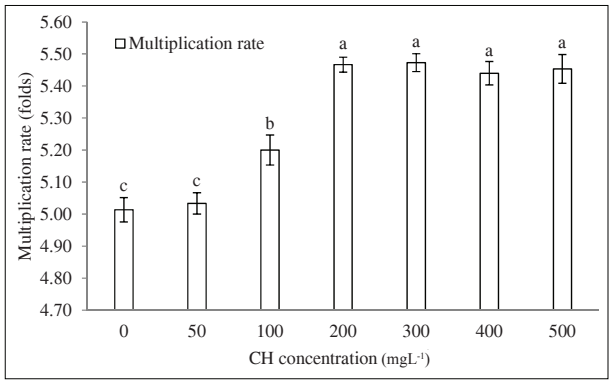

Figure 2: Effect of CH concentration on rate of multiplication (30 days of subculture). Different letters above bars show significant differences (DMRT, $p < 0.01$). Error bars indicate SE.

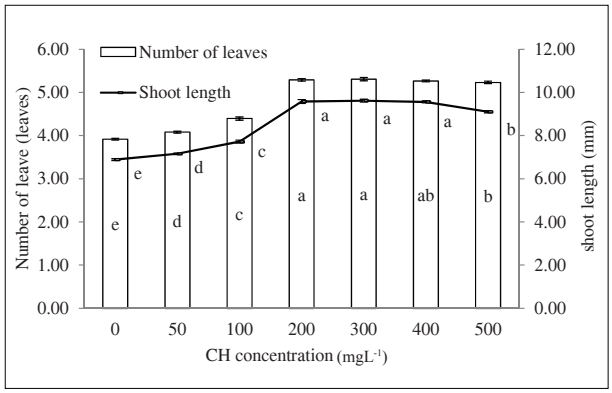

Figure 3: Effect of CH concentration on length and number of leaves per shoot (after 30 days of subculture). Different letters at centre bars or right lines show significant differences (DMRT, $p < 0.01$). Error bars, or line indicate SE.

Furthermore, the highest number of leaves per shoot was obtained in MSC media containing 200, 300, or 400 mgL^{-1} of CH compared to the MSC media containing other CH concentrations (50, 100, or 500 mgL^{-1}) ($p < 0.01$). Considering the best result of all parameters, MSC media containing CH of 200, 300 or 400 mgL^{-1} were recognised as optimum media for elongation and multiplication of shoots. Shoot clusters that developed in these media contained shoots with satisfactory shoot length, number of leaves per shoot as well as leaf shape.

Casein hydrolysate is a source of calcium, phosphate, several microelements, vitamins and most importantly, a mixture of up to 18 amino acids (George & De Klerk, 2008). Thus, the use of CH provides complementarily essential nutritional elements, which had been missed in the basal medium. Many authors reported that the use of CH could improve proliferation of *in vitro* shoots at the multiplication stage in many genotypes (Cohen & Cooper, 1982; Gao *et al.*, 2003; Chaturvedi *et al.*, 2004; Sridhar & Aswath, 2014; Hosny *et al.*, 2016). Prakash *et al*. (2003) reported that CH proved inhibitory for shoot differentiation in jojoba. In this study, MSC containing CH at concentrations of 200, 300 or 400 mgL^{-1} were recognised as optimum for shoot proliferation and similar results are reported in *Amorpha fruiticosa* (Gao *et al*., 2003) and neem (Chaturvedi *et al.*, 2004). In agreement with the results of the present study, Roy *et al.* (2012) claimed that addition of 200 mgL^{-1} CH to the multiplication medium of papaya increased the number of shoots per culture and shoot growth with healthy leaves. In contrast, Chan and Teo (1994) also used 500 mgL^{-1} CH for multiple shoots in 'Taiping' papaya.

Rooting of shoots

Response of papaya shoots to rooting in 1/2 MS or MS under different IBA concentrations after 30 days of culture are shown in Table 3. Both MS medium and IBA concentration affect root formation of *in vitro* shoots significantly ($p < 0.01$). Although there is no significant differences for percentage of shoots produced roots between 1/2 MS and MS media (Figure 4B), most of the other growth parameters such as number of roots

Table 3: Effect of different IBA concentrations on rooting of papaya shoot cultured on half and full strength MS medium (after 30 days of culture)

Media culture	IBA concentration (mgL^{-1})	Time required for root formation (days)	Rooting of shoot		
			Percentage of shoot produced root ± SE	No. of roots (roots) ± SE	Root length (mm) ± SE
MS/2	0.0	-	0.00 j	-	-
	0.5	14.90 ± 0.89 g	20.00 (26.33) ± 7.45 i	1.70 ± 0.45 f	28.07 ± 0.63 g
	1.0	12.33 ± 0.51 h	40.00 (39.16) ± 9.13 h	2.43 ± 0.09 de	31.42 ± 0.27 e
	1.5	10.82 ± 0.58 i	70.00 (56.97) ± 7.45 def	2.61 ± 0.23 cd	33.56 ± 0.44 b
	2.0	10.87 ± 0.18 i	100.00 (90.00) ± 0.00 a	5.50 ± 0.31 a	34.39 ± 0.37 a
	2.5	12.70 ± 0.22 h	100.00 (90.00) ± 0.00 a	5.57 ± 0.22 a	33.90 ± 0.22 ab
	3.0	17.75 ± 0.30 e	80.00 (63.67) ± 7.45 cd	5.38 ± 0.18 a	26.86 ± 0.18 h
	3.5	22.15 ± 0.56 c	73.33 (59.20) ± 9.13 de	3.92 ± 0.25 b	23.54 ± 0.26 k
	4.0	24.88 ± 0.39 b	63.33 (52.79) ± 7.45 efg	2.95 ± 0.37 c	20.39 ± 0.50 l
MS	0.0	-	0.00 j	-	-
	0.5	20.93 ± 0.38 d	36.67 (37.21) ± 7.45 h	2.27 ± 0.25 de	26.12 ± 0.40 i
	1.0	17.15 ± 0.36 e	50.00 (45.00) ± 11.79 gh	3.88 ± 0.30 b	30.05 ± 0.19 f
	1.5	16.34 ± 0.40 f	83.33 (68.49) ± 11.79 bc	3.89 ± 0.21 b	32.06 ± 0.11 d
	2.0	15.27 ± 0.30 g	100.00 (90.00) ± 0.00 a	3.87 ± 0.14 b	33.02 ± 0.11 c
	2.5	16.17 ± 0.21 f	89.33 (75.05) ± 9.83 b	3.81 ± 0.41 b	32.48 ± 0.10 d
	3.0	22.16 ± 0.44 c	70.00 (56.97) ± 7.45 def	2.99 ± 0.39 c	28.00 ± 0.20 g
	3.5	25.70 ± 0.30 a	56.67 (48.89) ± 9.13 fg	2.50 ± 0.17 cde	25.04 ± 0.22 j
	4.0	26.20 ± 0.87 a	40.00 (39.16) ± 9.13 h	2.07 ± 0.38 ef	23.57 ± 0.24 k

Figures in parentheses are transformed values by the angular transformation before statistical analysis. Means followed by the same alphabet are not significantly different based on DMRT (at $p < 0.01$).

and time taken for rooting were better in half strength MS medium than in full strength MS medium (Figure 4 A, C). The highest values for the time required for root formation, percentage of shoots that produced roots, number of roots and root length were obtained at 2.0 mgL^{-1} IBA concentration with values of 13.07 days, 100 %, 4.68 roots and 33.71 mm, respectively (Figure 5 A, B, C). Lowest values in most of the parameters were observed in the medium devoid of IBA ($p < 0.01$). When IBA was used at high concentrations, the time required for root formation was prolonged, number and length of roots were reduced and a large amount of calli was observed at the base of shoots (Figure 5 A, C; Figure 6 B, C). Interaction of MS and IBA revealed that adding IBA at 2.0 or 2.5 mgL^{-1} to half strength MS medium was best for rooting of papaya shoot compared to other combinations (Table 3, Figure 6 A). *In vitro* plantlets grew normally and vigorously in the potting medium containing peat moss : perlite (1:1) after greenhouse acclimation (Figure 7).

It is very difficult to induce a high percentage of rooting as well as produce high-quality adventitious root systems from *in vitro* shoots of *C. papaya* on an artificial medium (Winnaar, 1988; Drew & Miller, 1989). There are some complex methods applied for rooting of papaya shoots such as controlling aeration of the medium (Yu *et al.*, 2000) and treatment of shoots in media with PGRs before transferring to a root development medium without hormones (Panjaitan, 2007; Mumo *et al.*, 2013).

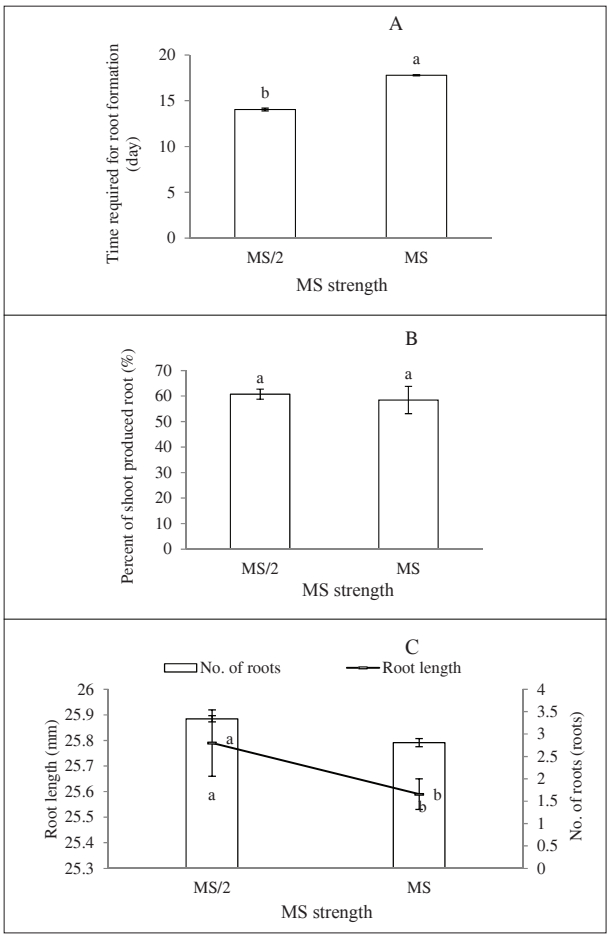

Figure 4: Effect of MS concentration on time required for root formation (A); percentage of shoots that produced roots (B) and no. of roots and root length (C). Different letters at centre bars or right lines show significant differences (DMRT, $p < 0.01$). Error bars, or line indicate SE

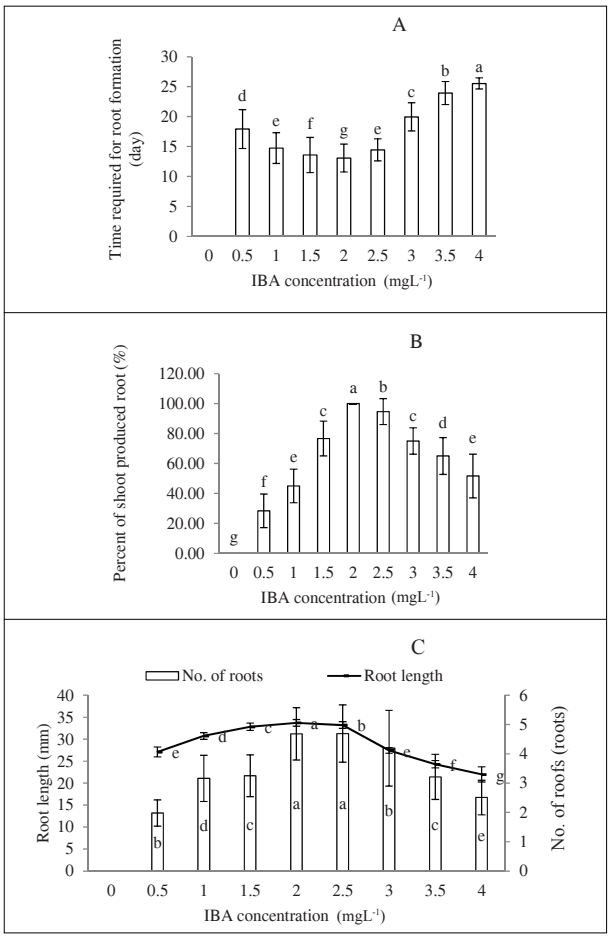

Figure 5: Effect of IBA concentration on time required for root formation (A); percentage of shoots that produced roots (B) and no. of roots and root length (C). Different letters at center bars or right lines show significant differences (DMRT, $p < 0.01$). Error bars, or line indicate SE

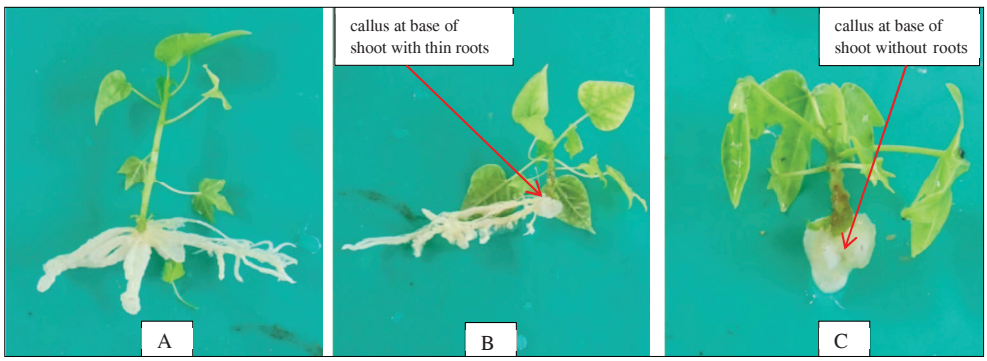

Figure 6: Rooting of micro-shoot in rooting media with 2.0 mgL^{-1} IBA (A); formation of callus at base of microshoot with thin roots (B) or without roots (C) from rooting media consisting of high IBA concentration

Figure 7: Acclimatised plantlet 40 days after transfer

Many authors adjusted the MS concentration for rooting of shoots and achieved different results. Wu *et al.* (2012) stated that 3/2 MS (1.5 times the macro-elements of MS) was optimum for root development. Chan and Teo (2002), Kabir *et al.* (2007) and Ahmed *et al.* (2007) used full- strength MS as basal rooting medium. Researchers popularly used half-strength macroelements of MS as basal culture medium for rooting micro shoots in papaya (Reuveni *et al.*, 1990; Yu *et al.*, 2000; Rohman *et al.*, 2007; Anandan *et al.*, 2011; Roy *et al.*, 2012; Ambasta & Kumari, 2013; Setargie *et al.*, 2015). In this study, better results for rooting of shoots were achieved on half-strength MS compared to full-strength MS medium. Adjusting the medium concentration to half-strength MS did not limit shoot growth and resulted in better rooting of shoots compared to full-strength MS. Yu *et al.* (2000) and Nhan (2004) agreed with this opinion when they tested different MS concentrations (1/4, 1/2, or 1/1 MS). This may be due to increased root initiation with reduced mineral concentration in the medium (Purohit *et al.*, 1994; Andrade *et al.*, 1999).

The role of auxins such as IBA, or NAA on *in vitro* rooting of shoots has been well documented (George *et al.*, 2008). IBA had greater ability to promote rooting compared to NAA in micropropagation of papaya (Winnaar, 1988; Roy *et al.*, 2012). Similarly, the present study also showed that IBA was essential for root initiation. Shoots did not form roots on media without IBA. In agreement with the results of Roy *et al.* (2012), high IBA concentrations produced less number of roots, and calli formation was observed at the base of shoots. According to this study, IBA at 2.0 mgL^{-1} was the optimum concentration for rooting of papaya shoots and similar results were reported by Hidaka *et al.* (2008), and Ambasta and Kumari (2013). These results were in agreement to those of Setargie *et al.* (2015) and Mumo *et al.* (2013), who reported supplementing 1.5 and 2.5 mgL^{-1} IBA, respectively to the rooting medium to obtain optimal rooting. However, some researchers stated that the best results for rooting was achieved at higher concentrations of IBA (4 or 5 mgL^{-1}) (Winnaar, 1988; Roy *et al.*, 2012), whereas Anandan *et al.* (2011), Yu *et al.* (2000) and Mohamad Fhaizal *et al.* (2006) claimed that the optimal root formation was found at a very low (0.5 mgL^{-1}) concentration of IBA. The variation of optimal IBA concentration in root formation endorsed the assumption that different papaya genotypes gave differing responses to PGR.

It has been described that an interaction was observed between IBA and medium concentrations on rooting by Rai *et al.* (2012) and Hiregoudar *et al.* (2003). The present study demonstrated that different IBA concentrations significantly affect rooting of 'Red Lady' papaya shoots cultured on half and full-strength MS medium and 2.0 or

2.5 mgL^{-1} IBA supplement to 1/2 MS medium recorded the best results for growth and rooting of shoots. Similar findings were reported by Ambasta and Kumari (2013) as well as Sekeli *et al.* (2013).

CONCLUSION

The present study describes an efficient *in vitro* technique for the rapid clonal propagation of 'Red Lady' papaya. The protocol describes efficient uses of growth regulators (BAP, kinetin, NAA, IBA), casein hydrolysate (CH) and MS concentrations for shoot regeneration from nodal explant, shoot multiplication and elongation, as well as rooting of *in vitro* shoots. Shoot regeneration was best under the influence of a combination of 0.5 or 1.0 mgL^{-1} BAP and 0.1 mgL^{-1} NAA. Combinations of 0.5 mgL^{-1} BAP, 0.1 mgL^{-1} NAA and 200 – 400 mgL^{-1} CH were effective for shoot multiplication and elongation. The best rooting response was achieved on half-strength MS medium supplemented with 2.0 or 2.5 mgL^{-1} IBA and plantlets were successfully acclimatised after 40 days. This successful protocol for micropropagation will contribute to the development of commercial clonal propagation programmes of papaya plants.

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