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In vitro Propagation of *Carica papaya* L. Variety 'Horana Papaya Hybrid 01' Using Shoot Tip

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Abstract *In vitro* plantlet regeneration ability of the local hybrid papaya variety Horana Papaya Hybrid 01 was evaluated using shoot tips from greenhouse-raised seedlings. Shoots tips surface sterilized in 20% Sodium Hypochlorite (Clorox®) for 20 min were established in Murashige and Skoog (MS) basal medium containing 6-Benzylaminopurine (BAP; 0.0,0.5,1.0,1.5 mg/L) in combination with 1-Naphthalene Acetic Acid (NAA; 0.0, 0.1, 0.5 mg/L) for shoot multiplication. Considering the high mean number of shoots per explant (4.8 ± 0.5) and absence of calli, 1.0 mg/L BAP was the best treatment for direct organogenesis. Proliferated shoots were transferred to a 1.5 strength MS medium containing 0.25 mg/L BAP and Gibberellic Acid (GA_3; 0.0, 0.15, 0.30 mg/L) for further elongation. Elongated shootlets were placed in half-strength MS medium supplemented with Indole-3-Butyric Acid (IBA; 0.0,1.0, 2.0, 3.0 and 4.0 mg/L) for rooting. The highest root induction response (86%) and roots suitable for acclimatization were observed with 2.0 mg/L of IBA. Plantlets were acclimatized in poly cups containing a sterile potting mixture (soil, sand, compost, and coir dust; 1:1:1:1) and a survival rate of 75% was achieved under *in vitro* conditions. The findings of the present study can be optimized to develop a suitable *in vitro* micropropagation protocol for rapid clonal propagation of this papaya variety for producing true to type planting material.

Keywords: *direct organogenesis, hybrid, micropropagation, papaya, shoot tip*

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1. Introduction

The papaya (*Carica papaya* L.) is a tropical fruit of family *Caricaceae*. Introduced to Sri Lanka in the 16th century, it is now a popular plantation crop giving high economic returns in the dry and intermediate zones [1]. However, papaya growers prefer imported hybrid (F_1) seeds for cultivation despite the availability of local germplasm, making local varieties less popular in local markets [2,3]. The 'Horana Papaya Hybrid 01' was released by the Department of Agriculture (DOA), Sri Lanka in 2014 to popularize local papayas among both farmers and consumers. It is the first F_1 papaya hybrid released in Sri Lanka and is a cross between two local inbred lines 'Rathna' and 'Cp -13' [3,4]. The hybrid offers several favorable agronomic traits such as high yield (nearly 50 fruits or 45 kg) per tree and offers moderate resistance to anthracnose and powdery mildew and the Papaya Ring Spot Virus (PRSC [4].

Papaya is a heterozygous, cross-pollinated crop, mainly propagated sexually through seeds. However, seed propagation leads to variability among offspring [1]. Thus, conventional breeding programs have been developed to obtain papaya lineages to be used in hybridization while focusing on heterosis (hybrid vigor). When two inbred lines are crossed**,** the resulting first generation (F_1) is uniform and vigorous in terms of morpho-agronomic characters, making them more suitable for commercial-scale cultivation. However, it consumes time, space, and resources to maintain such inbred lines [5].

Several papaya hybrids have been developed to have many desirable agronomic traits (such as yield, fruit size, quality, pest, and disease resistance). 'Known You Number One', 'Sun rise', 'Solo' and 'Red Lady'. are some popular imported F_1 hybrids cultivated in Sri Lanka [3]. Segregation of offsprings at the second filial generation (F_2) still causes variations in yield and quality parameters, which limits the stability of hybrids to the first (F_1) generation. Therefore an efficient clonal propagation method is necessary [6,7]. Asexual reproduction via grafting and rooted papaya cuttings has a low success rate. Thus, micropropagation is an effective alternative for producing disease-free, true-to-type plants from quality germplasm [1,5,7]. The use of pre-existing meristems (shoot tips and axillary buds) is the most common method for *in vitro* regeneration of papaya plantlets and is governed by many internal and external factors [8,9,10]. This includes the genotype of plants, explant type, and the

culture conditions (e.g. media constituents, light, temperature, and relative humidity) provided during micropropagation [11].

The present study aims at determining the effects of some selected plant growth regulators (PGRs) and culture conditions during stages of micropropagation (*in vitro* establishment, shoot multiplication, elongation root induction, and acclimatization) of 'Horana Papaya Hybrid 01' to produce tissue cultured plants via rapid clonal propagation.

2. Materials and Methods

2.1. Plant Material and Surface Sterilization

The F_1 seeds of 'Horana Papaya Hybrid 01' were obtained from the Plant Breeding Division, Fruit Research and Development Institute (FRDI), Horana, Sri Lanka. Seeds were soaked in 200 ppm of Gibberellic acid (GA_3) for 24 hrs. to enhance germination [12]. Four-day-old, germinated seedlings were planted in a potting mixture having soil: sand: compost: and coir dust (1:1:1:1) and maintained under greenhouse conditions to obtain explants. At the end of six weeks, shoot tips from greenhouse-raised F_1 seedlings were excised and proceeded for aseptic culture.

Shoot tips (1.5 cm) were washed under running tap water for 45 min. followed by rinsing in a 0.5% v/v liquid detergent solution. Afterward, they were immersed in 20% Clorox (Sodium Hypochlorite) for 20 min. with shaking (at 100 rpm) followed by rinsing twice with sterile distilled water to remove all traces of sterilant. Thereafter, the shoot tips were resized into 1 cm segments and cultured in twelve different multiplication media for shoot regeneration (Table 1).

2.2. Culture Media and Culture Conditions

Murashige and Skoog (MS) basal medium [13] with modified vitamins, 7.5 g/L agar, and 30 g/L of table sugar [2] were used in preparing culture media. The pH was adjusted to 5.8 before autoclaving. Changes made in the MS strength, sugar concentration and PGRs added have been mentioned under each instance. Cultures were placed in the growth room at 25±1°C for a photoperiod of 16 hrs. (cool white fluorescent lights at 2000 - 3000 lux intensity).

2.3. Shoot Multiplication and Elongation

Combinations of four levels (0.0, 0.5, 1.0, and 1.5 mg/L) of 6-Benzylaminopurine (BAP) and three levels (0.0, 0.1 and 0.5 mg/L) of 1-Naphthalene Acetic Acid (NAA) were used to determine the effect on *in vitro* shoot multiplication with eight replicates for each treatment (Table 1). Proliferating shoots were subcultured into the same respective media on the 4^{th} and 7^{th} weeks and allowed to multiply *in vitro* for 60 days [8].

An additional shoot elongation phase had to be carried out as the height of shoots produced in the above treatments was inadequate for rooting. At the end of 60 days, shoots from the three best proliferation media were selected for elongation after statistical analysis of shoot multiplication data. Treatments containing 1.5 strength MS basal media containing 0.0, 0.15, and 0.30 mg/L of Gibberellic acid (GA_3) in combination with 0.25 mg/L BAP were adopted as stated in Wu *et al.* [14]. Accordingly, individual shoots excised from proliferating clumps were placed in the above PGR combinations for 3 weeks for elongation.

2.4. Root Induction and Acclimatization

After elongation, root induction was done in a two-step procedure. First, the shoots were placed in a PGR-free MS medium for 10 days to remove the residual effects of previously used PGRs (BAP, NAA, and GA_3). Then, individual shoots were placed on half-strength MS media with five different levels of Indole-3-Butyric Acid (0.0,1.0,2.0,3.0, and 4.0 mg/L) for four weeks. *in vitro* root development was evaluated using three parameters namely, percentage of root induction, mean number of roots initiated per explant, and the mean root length.

Table 1. The Effect of BAP and NAA on the Mean Number of Shoots Produced Per Explant After 60 Days

Treatment code	BAP concentration (mg/L)	NAA concentration (mg/L)	Mean no. of shoots per explants ± SE
MS0		0.0	1.500± 0.342^{b}
N1*	0.0	0.1	2.250± 0.164^{b}
N2*		0.5	2.500± 0.342^{b}
B1		0.0	4.500± 0.957^{a}
SM1	0.5	0.1	3.375± 0.263^{a}
SM2*		0.5	5.333± 0.422^{a}
B2		0.0	4.750±0.479^{a}
SM3	1.0	0.1	3.375±0.460^{a}
SM4		0.5	4.125± 0.515^{a}
B3		0.0	3.625± 0.263^{a}
SM5	1.5	0.1	3.000± 0.267^{a}
SM6		0.5	4.500±0.378^{a}

Values are the mean number of shoots per explant from contamination-free cultures. Mean values having the same letter(s) are not significantly different by Tukey's multiple comparison test ($p < 0.05$) for BAP.
(SE= Standard Error, * Callus formation)

Following root induction, rooted plantlets were withdrawn from culture vessels and gently washed with sterile distilled water and planted in sterile polycups containing an autoclaved potting mixture of soil: sand: compost: coir dust (in 1:1:1:1 ratio). Each plant was irrigated with a PGR free full-strength liquid MS solution (10 mL), covered with polypropylene bags to maintain humidity, and placed in an air-conditioned room (25±1°C) that has access to sunlight while monitoring the number of surviving and dead plants regularly for a total of four (04) weeks [8,9,10].

2.5. Experimental Design

All *in vitro* experiments were arranged in a Completely Randomized Design (CRD). Each experiment (multiplication, elongation, and root induction) had at least seven replicates per treatment. Analysis of Variance (ANOVA) and Tukey's Pairwise comparison test was conducted for parametric data in significantly different ($p<0.05$) treatments. Nonparametric data were analyzed using the Kruskal Wallis test and were presented as the mean ± standard error (SE) using the MINITAB version 17.1.0 software [8,10].

3. Results and Discussion

3.1. Effect of BAP and NAA on Shoot Multiplication

Shoot proliferation was initiated after two weeks of *in vitro* establishment. By the fourth week, shoot multiplication was observed in all treatments. Skoog and Miller [15] reports that balancing auxin and cytokinin levels in the tissue culture medium affect organogenesis. Thus, different combinations of BAP and NAA were tested to identify the most suitable treatments [8,9,10]. Table 1 presents the mean number of shoots per explant produced by each treatment at the end of 60 days of incubation.

Results of the two-way ANOVA indicated that the interaction effect between BAP and NAA was not significant ($p=0.067$). However, BAP and NAA had a significant effect individually at $\alpha=0.05$ ($p=0.000$ and $p=0.000$). Thus, use of BAP and NAA were evaluated separately with one way ANOVA. Accordingly, all treatments containing BAP (0.5,1.0 and 1.5mg/L) alone or in combination with NAA, has significantly high multiplication rates (than ones without BAP). However, Tukey's pairwise comparison does not outline the best concentration among the tested BAP levels.

In addition to the statistical analysis. morphological traits (absence of callus formation and/or clumping of shoots) had to be considered when selecting suitable multiplication media. The use of BAP for shoot multiplication was preferred as it is a cost-effective cytokinin with widespread use and availability [8].

Callus induction was prominent in certain PGR combinations (Figure 1A) despite rapid multiplication. Such media are not suitable for producing true-to-type planting material. However, they can be used when indirect organogenesis is intended. Higher NAA levels (0.5mg/L) when combined with low cytokinin produced calli in treatment SM2 (0.5mg/L BAP + 0.5 mg/L NAA) whose auxin: cytokinin ratio (1:1) favors callus formation. Treatments N1 and N2 carrying NAA alone (0.1 mg/L and 0.5 mg/L of NAA) give low multiplication rates. Root initiation without any shoot proliferation was observed in N1 with 0.1 mg/L NAA.

Considering the high mean number of shoots (4.8±0.5) per explant and the absence of callus formation, 1.0 mg/L of BAP was the most effective treatment for direct organogenesis in the present study (Figure 1C). Even though higher BAP levels facilitate shoot proliferation, it is known to arrest individual shoot development in papaya [16,17]. In agreement with the above, treatments SM4 (1.0 mg/L BAP+ 0.5 mg/L NAA) and SM6 (1.5 mg/L BAP + 0.5 mg/L NAA) produced stunted shoot clusters with smaller leaves in the present study despite high multiplication rates (Figure 1B). Such shoots had to undergo a subsequent elongation phase.

In similar studies for papaya, Setargie *et al.* [18] and Panjaithan *et al.* [8] have obtained the highest mean number of shoots per explant in MS media with 1.0 mg/L BAP + 0.5 mg/L NAA and 1.0 mg/L BAP + 0.05 mg/L NAA for *Carica papaya* L. cv. Maradol and Eksotika respectively. An additional step of elongation with a lowered cytokinin level combined with various auxins (NAA, IAA) or GA_3 might be required for *in vitro* raised *C. papaya* shoots [8,19]. Even though GA_3 is known to cause shoot elongation, records on its applications in micropropagation of *C. papaya* are limited. Since the present study also had rapidly multiplying clumps of dwarf shootlets, an elongation step was necessary.

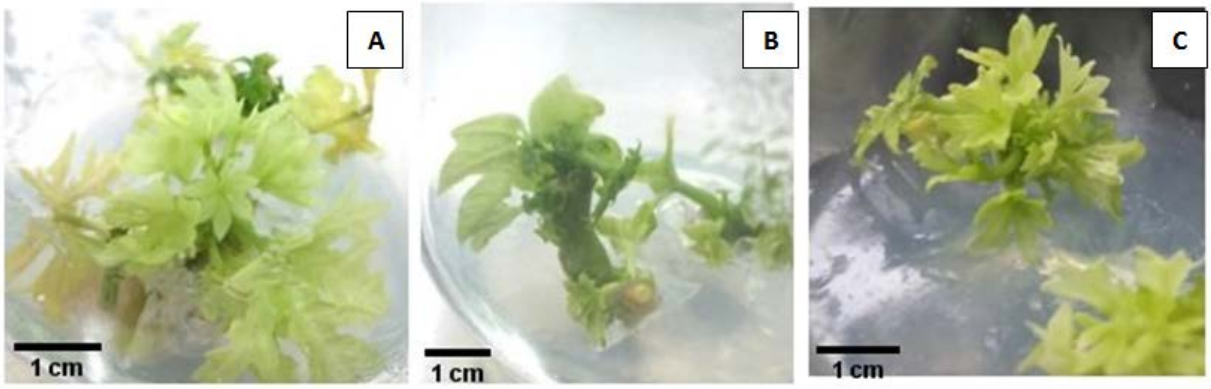

Figure 1. Results of i*n vitro* shoot multiplication of 'Horana Papaya Hybrid 01'. (A) Callus formation and pale colored shoots in 0.5 mg/L BAP + 0.5 mg/L NAA (B) Small , compact shootlets formed with 1.0 mg/L BAP + 0.5 mg/L NAA (C) Shoot formation in 1 mg/L BAP

3.2. Effect of GA_3 on Shoot Elongation

Gibberellins stimulate elongation of internodes promote meristematic growth under in *vitro* conditions [14,19]. Although findings of Wu *et al.* were adopted for shoot elongation, no significant height increments were obtained in the present study. Wu *et al.*, report that 1.5 strength MS basal medium containing 0.25 mg L^{-1} BAP and, 0.3 mg/L GA_3 cause significant height increments [14]. However, incorporating GA_3 into the multiplication phase itself (preferably after the first few subcultures) can be recommended [19,20] for this papaya variety to separate any compact shoot clumps before proceeding to rooting.

3.3. Rooting and Acclimatization

3.3.1. Effect of IBA on Root Initiation and Elongation

Since IBA is a synthetic auxin that is more stable than endogenous Indole Acetic Acid (IAA) present on adult papaya, it can be used for *in vitro* root induction in papaya [8]. The MS strength was reduced to half when preparing rooting media because lowering the mineral and nutrient supply is known to favor root induction [16,21].

All five treatments (including 0 mg/L IBA) showed rooting responses after four weeks. When the number of root initials per shoot was assessed using Kruskal Wallis nonparametric test, 4.0 mg/L IBA was revealed to be the best rooting treatment, followed by 2.0 mg/L IBA. The control treatment without IBA scored the least rank (Table 2). No significantly different treatment was found for the mean length of roots (at $\alpha = 0.05$) after conducting one-way ANOVA and Tukey's pairwise comparison (Table 2).

Table 2. Mean Number of Plants that Produced Roots, Mean Number of Roots Initiated Per Shoot, and the Mean Length of Roots Produced After Four Weeks of Incubation in Half-Strength MS Medium with Different IBA Concentrations

IBA (mg/L) on half MS	Mean no. of plants that produced roots ± SE	Mean no. of roots initiated per shoot ± SE	Mean length of roots (cm) ± SE
0.0	0.17 + 0.17 [a]	*0.33 ± 0.33 (10.1)	2.1 [a]
1.0	0.38 + 0.18 [a]	*1.00 ± 0.73 (13.0)	1.7 ± 0.3 [a]
2.0	**0.86 + 0.14 [a]**	***3.71 ± 1.44 (22.5)**	1.8 ± 0.2 [a]
3.0	0.71 + 0.18 [a]	*3.43 ± 1.25 (21.2)	1.9 ± 0.3 [a]
4.0	**0.86 + 0.14 [a]**	***4.71 ± 2.10 (22.8)**	2.0 ± 0.4 [a]

Mean values having same letter(s) in the second and fourth columns are not significantly different by Tukey's multiple comparison test ($p < 0.05$). Results in the third column (*) have been analyzed by Kruskal Wallis nonparametric test (values obtained for mean rank at 0.05 level of significance have been given within parenthesis).

The half-strength MS medium with 4.0 mg/L IBA produced the highest number of roots per shoot with 86% of root induction. However, the thin and hairy root initials were unsuitable for acclimatization (Figure 2A). Furthermore, rooting formation occurred from a mass of cells in the culture and not from the base of the explant. Therefore, half-strength MS medium with 2.0 mg/L IBA (which also had 86% root induction) can be considered suitable for *in vitro* root induction in 'Horana Papaya Hybrid 01' (Figure 2B and Figure 2C). Saker *et al.* [22] obtained the best root induction on full strength MS medium with 2.0 mg/L IBA for *C. papaya* variety 'Honey Dew'. Nguyen *et al* [23] reports successful root induction for 'Red Lady' papaya in both full and half-strength MS media when 2.0 - 2.5 mg/L of IBA was used.

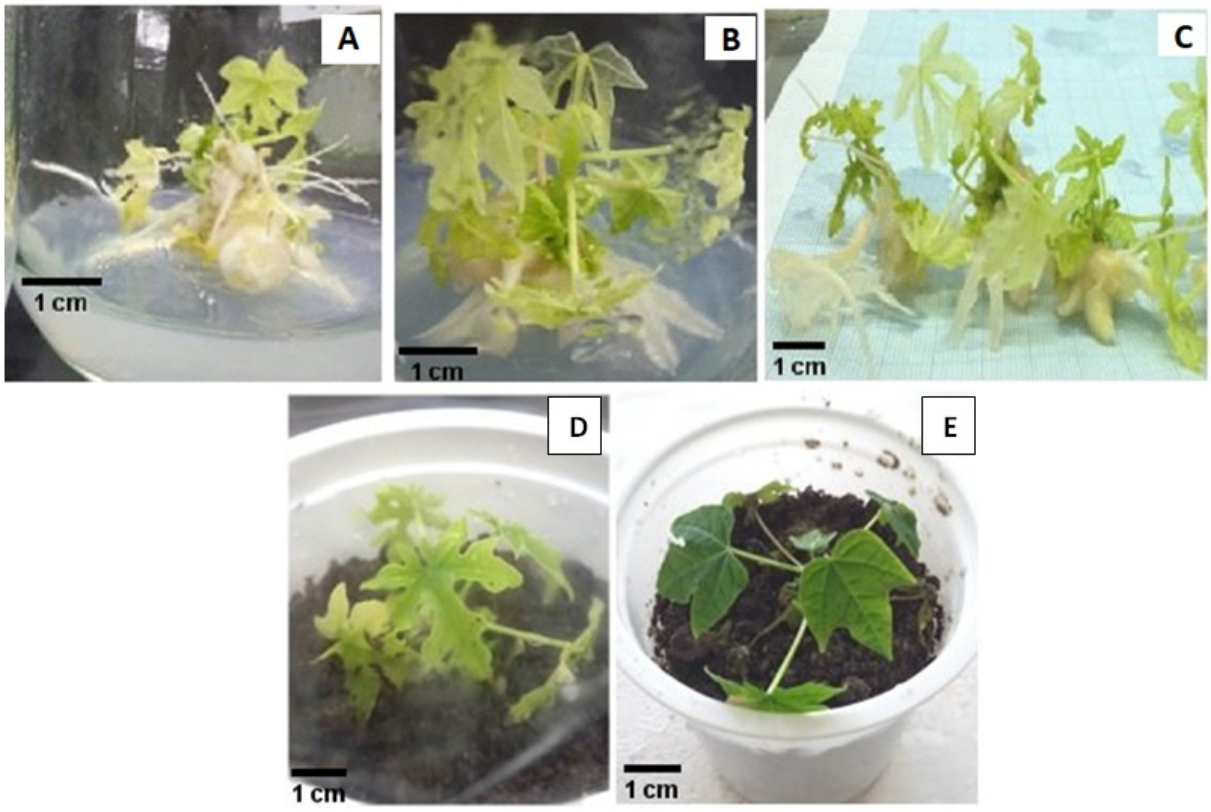

Figure 2. Formation of *in vitro* roots and acclimatization of ' Horana Papaya Hybrid 01'. (A) Root induction and callus formation in 4.0 mg/L IBA) (B) and (C) Root induction in 2.0 mg/L IBA. (D) An i*n vitro* raised plantlet of 'Horana Papaya Hybrid 01' after one week and (E) after four weeks of acclimatization

During acclimatization, 75% of the plantlets survived inside their propagators by the end of the fourth week (Figure 2D and Figure 2E). Due to time limitations, evaluation of *in vitro* acclimatized plants under field conditions could not be performed. Only 12.5% of plants survived under normal temperature and humidity when transferred to *ex-vitro* conditions. Hence, optimizing field establishment acclimatization phases is necessary.

4. Conclusion

Shoot tips are a suitable source of explants for *in vitro* propagation of *C. papaya* variety 'Horana Papaya Hybrid 01'. Shoot multiplication via Direct and indirect organogenesis can be achieved by altering the auxin: cytokinin ratio. Treatments with BAP only (1mg/L) or a higher BAP concentration in combination with NAA cause direct organogenesis. Increasing NAA levels, or combinations having lower BAP amounts leads to callus formation. Half strength MS with 2 mg/L IBA is the most suitable treatment for root induction. Further studies are necessary for investigating optimum conditions for root development and acclimatization. I*n vitro* raised propagules would also be a useful source of planting materials for future crop improvement programs aimed at breeding new varieties through protoplast culture, somatic embryogenesis, mutation breeding and genetic transformation [5,24,25].

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Statement of Competing Interests

The authors have no competing interests to disclose.

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