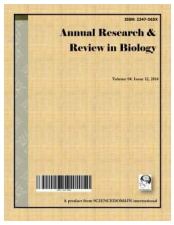

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In vitro Meristem Culture for Rapid Regeneration of Papaya Plantlets in Liquid Media

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Authors' contributions

This work was carried out in collaboration between all authors. Authors EKG and FKR designed the study, wrote the protocol and interpreted the data. Authors EKG and AWK anchored the field study, gathered the initial data and performed preliminary data analysis. While authors EKG, FKR and MMW managed the literature searches and produced the initial draft. All authors read and approved the final manuscript.

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ABSTRACT

Papaya (*Carica papaya L.*) is an important crop in Kenya, produced both for local consumption and export. Papaya propagation via seeds makes it difficult in differentiating gender at seedlings phase. However, *in vitro* system could offer a solution to this problem. This study was an attempt to determine rapid *in vitro* methods for regeneration of papaya plantlets using liquid and semi solid/liquid double layer media and different concentration of growth regulators. Shoot meristems were harvested from hermaphrodite 30 cm tall papaya seedlings raised in greenhouse and cultured in Murashige and Skoog basal media supplemented with 30 g^{-1} sucrose, and different concentrations of hormones which were treatments in liquid medium. The treatments comprised five levels (0, 0.05, 0.1, 0.15, and 0.2) of $mg.L^{-1}$ 2 Chloro 4 Pyridy – N Phenyl-urea (CPPU) in liquid and semi-solid/liquid double layer combination for shoot induction. For root initiation, four levels of Indole-3butyric acid (IBA) (0, 2.0, 2.5 and 3.0 $mg.L^{-1}$) were used. The experiments were laid out in

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a completely randomized design with 3 replicates. Number of shoots, shoot length and leaf number were recorded every 2 weeks for 8 weeks. The percentage number of shoots that produced roots was also recorded. There was significant differences between the liquid and semi-solid /liquid media and the level of hormones used on shoot induction. The highest number of shoots (10), shoot length and number of leaves was recorded in the semisolid/ liquid double layer combination with CPPU at 0.15 $mg.L^{-1}$. The highest root initiation (73.33%) was at 3.0 $mg.L^{-1}$ of IBA. Results indicated that semi-solid/liquid double layer combination could be used for rapid regeneration of papaya plantlets of known gender.

Keywords: Double layer culture; liquid media; Papaya; regeneration.

1. INTRODUCTION

Papaya belongs to the family *Cariacaceae* and originated from the lowlands of Central America ranging from Mexico to Panama [1]. It is widely distributed throughout the tropics and warmer subtropics of the world and has become naturalized in many areas including Kenya [2,3]. The papaya plant has the potential to produce fruits throughout the year.

In Kenya, papaya is produced by small and large scale farmers. However, there are many constraints which limit commercial production of papaya in most growing areas in Kenya. These includes the dioecious nature of papaya that makes it difficult to distinguish gender at seedling stage and the outbreak of papaya ring spot virus (PRSV) in most papaya-producing areas of the world has also not spared Kenya among others constraints [4,5].

In papaya, micropropagation has been attempted for a number of cultivars to solve some papaya production constraints such as producing plants of known gender [6,7,8]. A number of advantages reported in micropropagation of papaya that includes reduced time to produce new varieties, ease of maintaining genetic uniformity and production of plants of the same gender [9]. Agronomically, micropropagated trees have been reported to have a reduced juvenile phase than those raised through seeds and they produce fruits lower on the stem leading to early and higher yields [10,7,9]. Micropropagation by conventional techniques is typically a labor intensive time taking means of clonal propagation [11]. To overcome this, the use of shake cultures utilizing liquid culture medium has been promoted. The liquid medium allows the close contact with the tissue which facilitates the uptake of nutrients and phytohormones, leading to better shoot and root growth. Continuous shaking reduces apical dominance leading to induction and proliferation of numerous axillary buds. Further, with in the shake culture conditions, the growth and multiplication rate of shoots is enhanced by forced aeration, since continuous shaking of medium provides ample oxygen supply to the tissue which ultimately leads to their faster growth [11].

In vitro rapid shoot multiplication using liquid medium has been reported in clonal multiplication of elite sugarcane clones. Use of liquid culture reduces propagation cost and hence widening the scope of automation [12]. Liquid shake culture has also been used for rapid multiplication of mulberry plantlets with reduced labour and cost [13]. Use of liquid culture has been shown to promote rapid multiplication of shoots in other crop species. However, the use of liquid culture in papaya has not been reported in Kenya hence need to develop one.

2. MATERIALS AND METHODS

The study was conducted at Kenya Agricultural Research Institute (KARI) tissue culture laboratory in Kenya. Three popular local papaya lines were selected on the bases of high fruit yield, known mother plant gender and plant height were used in this study. Seeds were extracted and dried in a cool dry place. They were pre-treated by putting the seeds in a perforated plastic bottle wrapped with a wet paper towel and put in clear polythene bag for two weeks. They were then sown in trays with clean sand and watered regularly in the greenhouse. Pricking was done and seedlings were transplanted in polythene paper bags to provide stock plants. Meristems 1 cm shoot tips of three elite papaya lines were harvested from stock plants at 30 cm height and subjected to sterilization procedure as outlined below.

2.1 Sterilization Procedure

Meristems were thoroughly washed in running clean tap water for 1 hour followed by washing

with 1% savlon® and rinsed with distilled water. The meristems were soaked in systemic fungicide (Ridomil) ® at a concentration of 500 ppm for 1 hour and rinsed with double distilled water 3 times. The meristems were subjected in absolute alcohol for 30 seconds and rinsed with double distilled water before subjecting into 2% sodium hypochlorite and 2 drops of tween 20 for 15 minutes.

2.2 Shoot Induction and Development

The sterilized meristems were cultured in Murashige and Skoog basal media supplemented with 30 g^{-1} sucrose and two types of media [14]. The types of media were liquid medium and semi-solid double layer medium. The semi-solid/liquid double layer medium consisted of 5 ml semi-solid as the bottom layer and 10 ml liquid as the top layer. For liquid media, the meristem were placed in liquid while those for semi-solid/liquid double layer medium were inserted in the medium. These were mounted on an orbital shaker with speed of 60 rpm to initiate rapid shoot development. Each media had 5 levels (0, 0.05, 0.1, 0.15, and 0.2 $mg.L^{-1}$) of CPPU: Forchlorfenuron: N-(2-chloro-4-pyridyl)-N'-phenylurea.

They were put in a growth room at 25℃, 16 hour photoperiodic lighting for 8 weeks. The experiment was laid out in a completely randomized design with 3 replicates and 10 explants per treatment. Sub-culturing was done every two weeks to the same treatments. Number of shoots, their lengths and number of leaves were recorded every 2 weeks.

2.3 Rooting Induction

Shoots of 3 cm in length regenerated from double layer experiments were separated into individual explants using the method as described by Tsong with few modifications [15]. Briefly regenerated shoots were sub cultured in full strength MS containing 3% (w/v) sucrose, 0.28% (w/v) gerlite supplemented with Indole-3butyric acid (IBA) for root induction. Four concentration levels of IBA (0, 2.0, 2.5 and 3.0) $mg.L^{-1}$ were used and the explants placed in a dark room for one week and there after transferred into vermiculite supplemented with half strength MS supplemented with 3% (w/v) sucrose for further root development. The experiment was arranged in a complete randomized design (CRD) and was replicated three times with 20 explants per replication. The number of shoots that produced roots for every IBA concentration was recorded and expressed as a percentage. Data collected was analyzed using analysis of variance (ANOVA) and means differing were significantly were compared using students Newman Keuls (SNK) test at $P \leq 0.05$.

2.4 Hardening

Rooted plants were taken out carefully from the vermiculite containers and washed with clean tap water removing all the media remains. The plants were transplanted in pot measuring (9x6cm) containing forest soil, manure, sand and vermiculite in ratios of 2:1:1:1 respectively. The pots were covered with two layers shade net and kept moist with a mist propagator for one week. One layer of shade net was then removed and the second layer was removed on the second week. During the third week the plantlets hand already acclimatized and moved to the green house for further growth.

3. RESULTS

After five days in both media, new leaves started forming from the meristems. Breakage of leaves was witnessed in shoots cultured in liquid media. There was minimal leaf breakage of shoots cultured in semi-solid/liquid double layer media. After four weeks of culture, axillary buds emerged and elongated leaves from the shoot meristems developed for both media (Figs. 1a and b). Shoots in liquid media were succulent, transparent and thick in size a condition known as vitrification or hyperhydricity (Fig. 2).

There was no significant difference between the three papaya lines in response to the type of media and the level of hormones applied. There was, a significant interaction between the type of media and the concentration CPPU tested. In liquid medium 0.15 $mg.L^{-1}$ recorded the highest number of leaves per shoot with a mean of 4 and it was significantly different from other concentrations tested which had a mean of 2 leaves per shoot. In semi-solid/liquid double layer medium 0.15 $mg.L^{-1}$ CPPU had the highest number of leaves per shoot (10) followed by 0.1 $mg.L^{-1}$ (6) and 0.05 $mg.L^{-1}$ with 5 leaves per shoot. The least number of leaves was recorded in control and 0.2 $mg.L^{-1}$ with a mean of 2 leaves per shoot. The number of leaves induced from meristem was higher in the semi-solid/liquid double layer method than in liquid media (Table 1). There was also significant difference in the mean number of leaves in different concentration levels of CPPU (Table 1). As CPPU increased from 0.05 $mg.L^{-1}$ to 0.15 $mg.L^{-1}$ the number of

leaves increased from a mean of (2) in liquid to (4) and from (5) to (10) for semi-solid/liquid double layer. All other CPPU concentrations 0.05, 0.1, 0.2 and control recorded two leaves each. In a semi-solid/liquid double layer, 0.1 mg.L^{-1} of CPPU showed the highest number of leaves (10) followed by 0.1 (6) mg.L^{-1} and 0.05 mg.L^{-1} (5) respectively. 0.2 mg.L^{-1} and control had only two leaves each in semi-solid/liquid double layer (Table 1). In both media, 0.15 mg.L^{-1} of CPPU produced many leaves as compared to other concentrations.

Table 1. Effect of CPPU on the mean number of leaves in liquid and semi-solid/liquid double layer media approaches after eight weeks of culture

Concentration of CPPU (mg.L^{-1})	Liquid media	Semi-solid/ liquid double layer
	Number of leaves	Number of leaves
0	2.±0.34^{b}	2±0.21^{c}
0.05	2±0.24^{b}	5±0.35^{b}
0.1	2.±0.34^{b}	6±0.68^{b}
0.15	4.±0.51^{a}	10±1.36^{a}
0.2	2.±0.34^{b}	2±0.37^{c}

** Mean values within a column followed by same letters are not statistically different at P ≤ 0.05*

3.1 Shoot Length

After eight weeks of culture, there was significant difference in the mean shoot length between the two methods (Table 2). In liquid medium, 0.15 mg.L^{-1} of CPPU produced the longest shoot length (1.567) followed by 0.1 mg.L^{-1} (0.767) and 0.2 mg.L^{-1} with (0.711) respectively. However there were no significant differences in other concentrations in liquid medium (Table 2). In semi-solid/liquid double layer approach, 0.15 mg.L^{-1} of CPPU produced the longest mean shoot length of (1.667) cm followed by 0.1 mg.L^{-1} 1.069 cm. There were no significant differences between the liquid and semi-solid/liquid double layer methods in shoot length induction after eight weeks of culture (Table 2).

3.2 Shoot Number

There were significant differences in the concentration of CPPU on shoot induction using liquid and semi-solid/liquid double layer approach (Table 3). In the liquid media there was more explants suffering hyperhydricity than the semi-solid/liquid double layer method. In liquid medium, the highest mean number of shoots [3] was recorded at 0.15 mg.L^{-1} of CPPU. While other concentrations recorded same number [2] of leaves.

In semi-solid/liquid double layer, 0.15 mg.L^{-1} of CPPU recorded the highest number of shoots [10] followed by 0.1 of mg.L^{-1} CPPU [6] and 0.05 mg.L^{-1} [4]. There was a significant difference between the number of shoots in 0.15 mg.L^{-1} of CPPU in liquid and semi-solid/liquid double layer. The semi-solid/liquid double layer had the highest number of shoots as compared to liquid medium (Table 3). At 0.15 mg.L^{-1} in the semi-solid/liquid double layer, the explants did not suffer hyperhydricity, leaves breakages or chrolosis but explants appeared more vigorous.

Fig. 1a. Shoot induction in liquid media 4 wks

Fig. 1b. Shoot induction in semi-/liquid double 4 wks

Fig. 2. Succulent, transparent and thick 4 wks 4 shoots

3.3 Root Induction

The percentage of rooted plants was significantly different among the IBA concentrations tested. IBA concentrations at 3 mgl^{-1} produced the highest number of rooted plants of (73.33%) while 1 mgl^{-1} of IBA had the lowest number of rooted plants of (33.33%). Shoots in media without IBA did not produce any root (Fig. 3).

4. DISCUSSION

In this study an *in vitro* regeneration system of three papaya lines using their shoot meristems and two media methods, liquid and semi solid/liquid, was attempted. Hyperhydricity of micropropagated shoots, formerly called verification, was evident in liquid medium treatment. This could be caused by culture medium which has been reported to cause vitrification of tissues. According to work reported, reduction agar or lack of it in the culture medium causes vitrification during the growth of the tissues [16].

Table 2. Effect of CPPU on Shoot length using liquid and semi-solid/liquid double layer media approaches after eight weeks of culture

Concentration of CPPU ($mg.L^{-1}$)	Liquid	Semi solid double layer
	Shoot length (cm)	Shoot length (cm)
0	0.667 ± 0.06^{b}	0.778 ± 0.05^{c}
0.05	0.656 ± 0.04^{b}	0.592 ± 0.04^{c}
0.1	0.767 ± 0.07^{b}	1.069 ± 0.10^{b}
0.15	1.567 ± 0.11^{a}	1.667 ± 0.19^{a}
0.2	0.711 ± 0.06^{b}	0.667 ± 0.08^{bc}

** Mean values within a column followed by same letters are not statistically different at P ≤ 0.05*

The semi-solid/liquid double layer experiment had minimal breakages of leaves and shoots during shaking. The semi-solid liquid double layer had higher number of shoots, leaves and shoot length than liquid media in the same media CPPU concentration. The probable reason for minimal breakages in semi solid/liquid double layer media was that the shoots and leaves were stable during the shaking. There is also a study that reported reduced breakages of shoots and leaves when double layer was used to stabilize shoots in avocado liquid medium culture [17].

There was no significant difference between the three papaya lines in response to the type of media and the level of growth regulators applied in the mean number of shoots, number of leaves and shoot length. These results are supported by research done which showed a limited genetic variation among the papaya accessions in Kenya [18]. This could be the reason of no significant differences among papaya lines tested in this study.

Table 3. Effects of CPPU on number of shoots using liquid and semi-solid/liquid double layer approaches after eight weeks of culture

Concentration of CPPPU ($mg.L^{-1}$)	Liquid	Semi-solid/ liquid double layer
	Number of shoots	Number of shoots
0	2 ± 0.20^{b}	2 ± 0.22^{c}
0.05	2 ± 0.20^{b}	4 ± 0.36^{c}
0.1	2 ± 0.35^{b}	6 ± 0.67^{b}
0.15	3 ± 0.32^{a}	10 ± 1.36^{a}
0.2	2 ± 0.25^{b}	2 ± 0.32^{c}

** Mean values within a column followed by same letters are not statistically different at P ≤ 0.05*

Different concentrations of CPPU tested influenced the number of shoots, number of leaves and the shoot length. In both methods, increase in concentration of CPPU up to 0.15 $mg.L^{-1}$ increased the number of shoots, leaves and shoot length per explant. This corroborates with report which indicated that more shoots were obtained when cytokinine was increased [19]. However the mean number of shoot, leaves and shoot length decreased as the CPPU concentration increased to 0.2. $mg.L^{-1}$ The most probable reason would have been that 0.15 $mg.L^{-1}$ is the optimum concentration for shoot induction and development in the liquid media methods used. Excessive concentration of cytokinin has been reported to inhibit shoot growth and reduce reduced proliferation rates in papaya [10].

The influence of various concentrations i.e., 0.0, 1, 2, 2.5 and 3 $mg.L^{-1}$ of IBA on root formation of the *in vitro* derived shoots of papaya were investigated. Data presented in Fig. 3 indicated that addition of IBA in concentration of 3 $mg.L^{-1}$ /l showed the best results of root formation (73.33%). These results are closer to others where, 4 $mg.L^{-1}$ of IBA was found to be more effective on *in vitro* rooting of papaya [20]. However, other researchers used medium containing 2 $mg.L^{-1}$ IBA for *in vitro* rooting of papaya [10,21]. An other study conducted found that, NAA at 1 $mg.L^{-1}$ was most optimum for *in vitro* papaya rooting [22]. The difference between these findings and those reported by other authors could be due to genotypic differences.

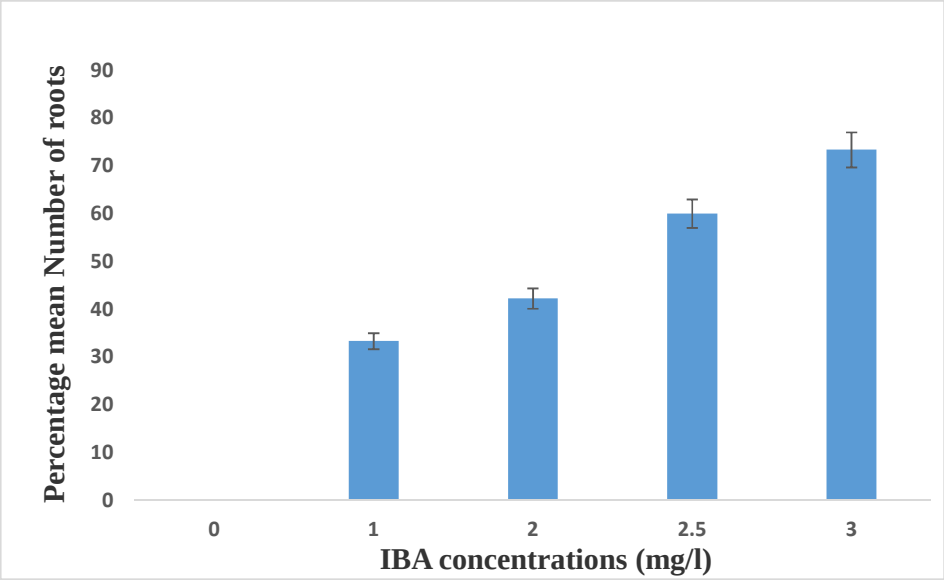

Fig. 3. Effects of different concentration of IBA on root induction on full strength MS medium

5. CONCLUSION

The shoot induction proliferation and rooting in this study is an indicator that semi-solid double layer technique can be used for rapid generation of papaya plantlets of known gender. With semi-solid double layer technique the shoot growth and development was doubled indicating that the regeneration of papaya in the media can be enhanced Semi-solid double layer media combination or (bi-layer) at CPPU hormone level of 0.15 $mg.L^{-1}$ is more efficient in shoot multiplication and development. Further work is recommended to optimize media for shoot elongation and rooting.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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