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PROPAGATION OF SAPOTA (*CASIMIROA EDULIS*) TREES BY USING TISSUE CULTURE TECHNIQUE

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A promising method for *in vitro* propagation of white sapota (*Casimiroa edulis* L.) was established. Shoot tips and nodal segments of *C. eduils* were cultured on Murashige and Skoog (MS) medium supplemented with 2.22 µM 6-benzylamino purine (BAP) and 1.07µM ß-naphthalene acetic acid (NAA) recorded the maximum growth percentage of 88.88 and 77.77%, and average shoot length of 2.76 and 2.4 cm, for shoot tips and nodal segments, respectively. Regarding shoots multiplication rate, it was significantly affected by the concentration of BAP, as 6.77 shoots /explant were recorded using MS medium containing 13.32 µM BAP + 4.90 µM N6-2- isopenteny adenine (2iP). For rooting, half and full strength MS medium supplemented with 0.00, 2.46, 4.90, 9.80, 19.60 and 39.20 µM indol-3-butyric acid (IBA) in combination with 0.00 and 2.69 µM NAA were examined. The highest rooting percentage, average number of roots/shoot and average root length (44.44 %, 1.88 and 2.46 cm, respectively) were obtained on half strength MS medium containing 19.60 µM IBA and 2.69 µM NAA. Finally, plantlets were successfully acclimatized to greenhouse conditions and grew vigorously with no apparent phenotype aberrations.

Keywords: Rutaceae, micropropagation, nodal segments, shoot tips

White sapota (*Casimiroa edulis* L.) is a species of tropical fruiting tree from the family Rutaceae, native to Eastern Mexico and Central America South to Costa Rice (Murillo et al., 2007). It is an evergreen fruit tree (Yonemoto et al., 2007) and also known as matasano, zapota, or celanco (Yahia, 2005). The plant can reach 15 m, propagated by seeds; the fruit reaches 4 to 10 cm in diameter, each with white seeds (2.5 – 5.0 cm long and 1.25 – 2.5 cm thick). Its harvest time is indicated by changes in color from green to yellow (Donadio et al., 1998). It is well known that white sapota's seeds possess medicinal properties, which have yielded the identity of many pharmacologically active compounds, including: n-

methylhistamine, n-dimethylhistamine and histamine (Murillo et al., 2007). White sapota cultivars display distinct differences in flowering, fruit set, fruit yield as well as other fruit characteristics; i.e. size, shape, skin and flesh color, brix levels and seed fresh ratios (Yonemoto et al., 2007). The plant readily adapts to subtropical climate, such as in the United States, Mexico in North America and in Egypt (Nerd et al., 1992). Such trees are moderately tolerant to drought, but not tolerant to wet soil conditions. In addition, white sapota is resistant to phytophthora and armilaria (Yahia, 2005). It is important to note that in Egypt, white sapota is commonly propagated by seeds (Morton, 1987) resulting in great genetic and fruiting variations amongst trees (Abo-El-Ez et al., 2013). Whereas, vegetative propagation is considered the most important method to obtain true to type plants. Micropropagation of white sapota is recommended, because by *in vitro* techniques, the number of plants produced would not be limited by seeds supply and also more uniform plant populations might be produced (Moore et al., 1992). Besides, there are no available data in the literature on the *in vitro* propagation of white sapota compared with the other species of family Rutaceae. This study is the first to be reported regarding *in vitro* propagation of white sapota. In addition, the data would be helpful in developing a protocol to make *in vitro* propagation of white sapota a feasible alternative to conventional propagation for the commercial production of the plant. In Egypt, this fruit tree is rare and there are only few trees, which needed to be conserved and recultured. Therefore, the present study aims at developing an efficient *in vitro* propagation system for rapid and mass propagation of white sapota (*Casimiroa edulis* L.) plant.

MATERIALS AND METHODS

1. Explant Source and Sterilization

Shoot tips and nodal segments of healthy lateral branches of white sapota were collected from adult mother plants aged 30 years old and grown in Faculty of Agriculture, Al-Azhar University, Cairo, Egypt. These explants were placed under running tap water for two hours followed by washing with commercial detergent for five minutes, then washed with tap water for one hour. Surface sterilization was done by immersing explants in 1.5 and 2.5% sodium hypochlorite solution containing two drops of tween 20 for 15 and 30 min, for shoot tips and nodal segments, respectively. Finally, explants were rinsed five times with sterile distilled water.

2. Culture Establishment

The explants were cultured on basal MS medium (Murashige and Skoog, 1962) (Duchefa, Haarlem, the Netherlands) supplemented with 6-benzylamino purine (BAP) at concentrations of 2.22, 4.40, and 6.66 µM and ß-naphthalene acetic acid (NAA) (Sigma Cell Culture, St. Louis, USA), at a

concentration of 1.07 μM, in addition to the control MS medium without PGRs. Sucrose at 30 g/L and 100 mg myo-inositol were added. The pH of the medium was adjusted to 5.7 – 5.8, then was solidified with 3 g/L phytagel (Duchefa, Haarlem, the Netherlands). Media were dispensed in 25x150 mm culture tubes (Sigma Cell Culture, St. Louis, USA) before autoclaving under 1.05 kg/cm^2 pressure at 121C^o for 20 min. Cultures were incubated in a growth chamber at 25$\pm$1 C^o, 16:8 h photoperiod and 3000 Lux illumination intensity with white fluorescent light. After six weeks, survival percentage (%), growth percentage (%) and average shoot length (cm) were recorded.

3. Shoot Multiplication

The shoots were multiplied on MS medium supplemented with different concentrations of BAP (4.40, 8.90, 13.32, 17.76 and 22.20 μM) in combination with 4.90 μM 2-isopentenyle adenine (2iP) (Sigma Cell Culture, St. Louis, USA). In addition to control MS medium without PGRs. Proliferation rate (average number of new shoots produced per explant) and average shoot length (cm) were recorded after four weeks of culturing. The micropropagation cycle consisted of a monthly subculture of shoots.

4. Rooting Induction and Acclimatization

Elongated shoots were cultured on solid half and full strength MS medium containing indol-3-butyric acid (IBA) (Sigma Cell Culture, St. Louis, USA) at 0.0, 2.46 , 4.90, 9.80, 19.60 and 39.20 μM and NAA (0.00 and 2.69 μM). The cultures were maintained for eight weeks and incubated at 25$\pm$ 1°C. The percentage of rooted shoots (%), average number of roots/ shoot and average root length (cm) were evaluated. The rooted shoots were removed from the culture tubes, washed with tap water to remove adhering medium, dipped carefully for 10 min in 1% bavistin (systemic fungicide) and transferred to plastic pots with a mixture of sand, garden soil and peat moss (1:1:1 v:v:v). Initially, plantlets were covered with a polyethylene film, which was gradually eliminated in six weeks. The pots were placed in greenhouse for completing their acclimatization. The plantlets were irrigated with ½ MS medium for eight weeks in greenhouse (28 $\pm$ 2°C, 70 – 80% relative humidity).

5. Statistical Analysis

Experimental data were conducted as completely randomized design, with 45 replicates for each treatment. Variance analysis of data was carried out using ANOVA program for statistical analysis. The differences among means for all treatments were tested for significance at 5% level by using Duncan (1955) and modified by Snedecor and Cochran (1980).

RESULTS AND DISCUSSION

1. Establishment Stage

Concerning the establishment of white sapota, data in table (1) show that growth percentage to survival ranged between 66.66 to 88.88% and 55.55 to 77.77%, for shoot tips and nodal segments, respectively. While, average shoot length ranged from 1.33 to 2.76 cm and 1.04 to 2.40 cm, for shoot tips and nodal segments, respectively. Data obtained after six weeks from culturing revealed that shoot tips and nodal segments gave the highest growth percentage (88.88 and 77.77%, respectively) and average shoot length (2.76 and 2.40 cm, respectively) on MS medium supplemented with 2.22 µM BAP + 1.07 µM NAA (Fig. 1 A).

Table (1). Effect of explant type and growth regulators (BAP and NAA) on *in vitro* establishment of white sapota.

BAP (µM)	NAA (µM)	Growth %		Average shoot length (cm)	
		Shoot tip	Nodal segment	Shoot tip	Nodal segment
0.00	0.00	66.66b	55.55c	1.33c	1.04d
0.44	1.07	55.55c	1.67c	1.15d	1.15d
2.22	1.07	77.77a	2.76a	2.40a	2.40a
4.44	1.07	66.66b	1.93c	2.10b	2.10b
6.66	1.07	55.55c	2.01b	1.90c	1.90c

Means in each column having the same letters are not significantly different at 5% level.

However, the shoot tips when cultured on MS medium containing 6.66 µM BAP + 1.07µM NAA gave 66.66% of growth and 2.01 cm shoot length. On the other hand, shoot tips were cultured on MS medium supplemented with BAP (0.00, 0.44 or 4.44 µM) + NAA at 1.07 µM gave the same results of growth percentage (66.66%) and different average shoot length of 1.33, 1.67 and 1.93 cm, respectively. Concerning nodal segments, they gave 77.77% of growth and the average shoot length of 2.40 cm when cultured on MS medium containing 2.22 µM BAP + 1.07 µM NAA. The growth percentage had insignificant difference when nodal segments cultured on MS medium containing BAP (0.00, 0.44 or 6.66 µM) + NAA at 1.07 µM, it reached 55.55%. The same media gave shoot length of 1.04, 1.15 and 1.90 cm, respectively. From the previous results it is clear that BAP and NAA combinations gave promising establishment results for white sapota explants. These results are in agreement with those obtained by Perez-Molphe-Balch and Ochoa-Alejo (1997) who found that BAP and NAA are more effective than BAP alone on *Citrus reticulata*. Cellular differentiation and organogenesis in tissue and organ cultures have been found to be

controlled by an interaction between cytokinin and auxin concentrations. It is apparent that not only auxin and cytokinin levels, but the proportions of one to other are determinants for cell cycle, cell division and differentiation control. Cell division seems to be regulated by the joint action of auxins-cytokinins, each of which appears to influence different phases of cell cycle. Generally, the composition of basal medium, the category and concentration of PGRs, and their combinations are key factors influencing adventitious shoots induction (Feng et al., 2010).

2. Shoot Multiplication

In order to increase the number of shoots per explant, *in vitro* shoots were cultured on MS medium supplemented with BAP at 4.44, 8.90, 13.32, 17.76 and 22.20 µM and 4.90 µM 2iP, in addition to the control treatment. Data in table (2) show that shoot number was affected by BAP concentration.

Table (2). Effect of cytokinins (BAP and 2iP) on shoot multiplication of white sapota.

Concentration (µM)		Average number	Average shoot
BAP	2iP	of shoots/explant	length (cm)
0.00	0.00	2.44 d	2.31 d
4.44	4.90	3.44 c	4.94 a
6.90	4.90	5.00 b	3.98 c
13.32	4.90	6.77 a	4.08 b
17.76	4.90	5.22 b	3.50 c
22.20	4.90	3.66 c	2.10 d

Means in each column having the same letters are not significantly different at 5% level.

Shoot multiplication rate ranged from 2.44 to 6.77 shoots/explant. The maximum number of shoots (6.77 shoots/ explant) were recorded on MS medium supplemented with 13.32 µM BAP + 4.90 µM 2iP (Fig. 1 B), followed by MS media containing 17.76 µM BAP + 4.90 µM 2iP and 6.90 µM BAP + 4.90 µM 2iP, which gave 5.22 and 5.00 shoots/ explant, respectively. On the other hand, MS medium without PGRs gave the least average number of shoots (2.44 shoots). Regarding the average shoots length, it was ranged from 2.10 to 4.94 cm. MS medium supplemented with BAP and 2iP at 4.90 µM, for each was the best concentration for shoot length (4.94 cm). It was noticed that shoot length decreased by increasing BAP concentration. Similar results were observed by Carimi and Pasquale (2003), who found that BAP at different concentrations has been the most commonly used cytokinin for multiplication of *Citrus* shoots. In addation, using BA and 2iP were useful. 2iP was most commonly selected because of its lower cost, but in some species, mixtures of the two compounds may give

better results than either compound alone (Eccher and Non, 1989). These results clear the mode of action of cytokinins on stimulation of both cell division and growth promotion of axillary shoots in plant tissue culture as reported by Gray et al. (2005) and George et al. (2008).

3. Rooting and *Ex vitro* Acclimatization

Low rooting efficiency is a major problem in the *in vitro* production of white sapota. It is inferred from the data in table (3) that half strength MS medium supplemented with 19.60 µM IBA + 2.69 µM NAA gave the highest rooting percentage, number of roots/ shoot and average root length of 44.44%, 1.88 and 2.46 cm, respectively (Fig. 1C). Decreasing or increasing IBA concentration than 19.60 µM either in half or full strength MS medium, decreased the percentage of rooting, average number of roots/shoot and average length of roots.

Table (3). Effect of half and full strength MS medium supplemented with different concentrations of IBA and 2.69 µM NAA on *in vitro* rooting of white sapota.

MS Strength	Concentration (µM)		Percentage of rooted shoots (%)	Average no. of roots/shoot	Average root length (cm)
	IBA	NAA			
Half	0.00	0.00	00.00 e	0.00 d	0.00 e
"	2.46	2.69	11.11 d	0.22 c	1.40 c
"	4.90	2.69	33.33 b	0.78 b	1.69 b
"	9.80	2.69	33.33 b	0.88 b	1.88 b
"	19.60	2.69	44.44 a	1.88 a	2.46 a
"	39.20	2.69	33.33b	0.88 b	2.07 a
Full	0.00	0.00	00.00 e	0.00 d	0.00 e
"	2.46	2.69	00.00 e	0.00 d	0.00 e
"	4.90	2.69	11.11 d	0.22 c	0.85 d
"	9.80	2.69	22.22 c	0.44 c	1.13 c
"	19.60	2.69	33.33b	1.00 b	1.17 c
"	39.20	2.69	22.22 c	0.55 c	1.24 b

Means in each column having the same letters are not significantly different at 5% level.

While, no rooting was observed when shoots were cultured on half and full strength MS medium without any auxins and full strength medium with IBA and NAA at 2.69 µM for each. Difficulties in inducing roots have been found in the most of species, like walnut (McGranahan et al., 1988) and *Carrizo citranga* (Moore et al., 1992) and have resulted in relatively low production of regenerated plants. Pena et al. (1995) worked on sweet orange and got only 3.2% rooting. Also, medium which containing IBA was

important for the roots formation in many different *Citrus* species (Carimi and Pasquale, 2003). Thus, the presence of an auxin is generally necessary to promote rooting in *C. reticulata* (Chayanika et al., 2011). IBA is commonly used to promote of *in vitro* root initiation (Pan and Zhao, 1994). IBA may also enhance rooting *via* increasing internal free IBA or may synergistically modify the action of endogenous synthesis of IAA (Krieken et al., 1993).

Concerning the acclimatization, rooted plantlets were successfully acclimatized, when they were transferred to pots containing a mixture of sand, garden soil and peat moss (1: 1: 1: v:v:v) in greenhouse conditions. About 30% of plants were successfully established in and out greenhouse (Fig. 1D, E and F).

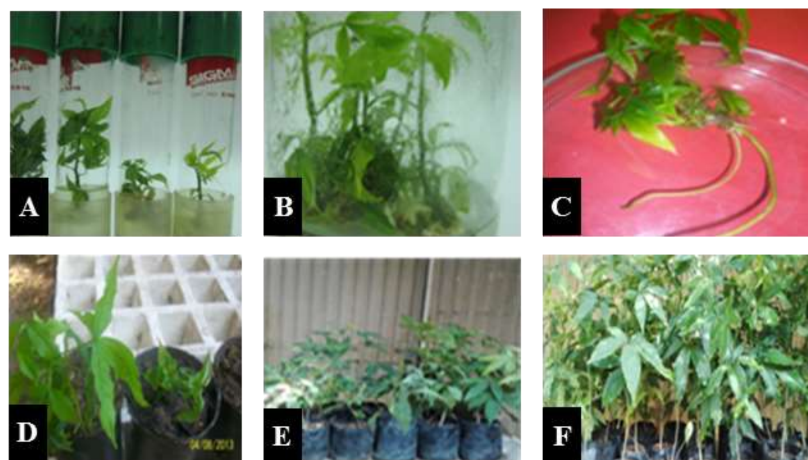

Fig. (1). Micropropagation stages of white sapota (*Casimiroa edilus* L.); **(A)** Establishment stage, **(B)** Multiple shoots, **(C)** *in vitro* rooted shoot, **(D)** acclimaticed plantlets in greenhouse after one month, **(E)** acclimatized plantlets out of greenhouse after six months and **(F)** acclimatized plantlets out of greenhouse after twelve months.

CONCOLUSION

This is the first report describing reproducible protocol for micropropagation of white sapota (*Casimiroa edulis* L.) using shoot tips and nodal segments. MS medium supplemented with 2.22 µM BAP and 1.07 µM NAA gave maximum growth percentage and average shoot length for both explants. While, the highest shoots multiplication rate was obtained on MS medium containing 3.0 µM BAP + 4.90 µM 2iP. For rooting, half strength MS medium supplemented with 19.6 µM IBA and 2.69 µM NAA was

effective for rooting. The *in vitro* grown plantlets were successfully acclimatized to greenhouse conditions. In conclusion, this work needs more experiments to increase rooting percentage and number of roots/ shoot, but an ideal and successful protocol for micropropgation of white sapota was presented.

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إكثار أشجار السبوتا بإستخدام تقنية زراعة الانسجة

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تهدف هذه الدراسة إلى الإكثار المعملي لنبات السبوتا، حيث تم زراعة البراعم القمية والأجزاء الساقية البرعمية على بيئة موراشيجي وسكوج (MS) مضافاً إليها ٢,٢٢ مولر بنزيل أمينو بيورين (BAP) و١,٠٧مولر نفثالين حمض الخليك (NAA)، حيث سجل أعلي نسبة نمو ٨٨,٨٨ و٧٧,٧٧٪ ومتوسط طول الأفرع ٢,٧٦ و٢,٤٠ سم، لكلًا من البراعم القمية والأجزاء الساقية، على التوالي. أما بالنسبة لمرحلة التضاعف سجل ٦,٧٧ فرع/جزء نباتي وذلك باستخدام بيئة MS موراشيجي وسكوج مضافًا إليها ١٣,٣ مولر BAP و٤,٩٠مولر NAA. عند زراعة الأفرع على نصف قوة بيئة MS مضافًا إليها١٩,٦٠مولرمن أندول حمض البيوتريك (IBA) و٢,٦٩مولر NAA أعطت أعلى نسبة تجذير، متوسط عدد الجذور ومتوسط طول الجذور ٤٤,٤٤٪ ، ١,٨و٢,٤٦ سم، على التوالي . بالنسبة لمرحلة الأقلمة تم إنتاج نبتات قوية وناجحة ولا يوجد بها أى تغيرات في المظهر الخارجي وذلك تحت ظروف الصوبة.