An Improved Protocol for Micropropagation of Jojoba (*Simmondsia chinensis* (Link) Schneider)

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Received: 22/4/*2015*

Abstract: *In vitro* consecutive micropropagation stages of *Simmondsia chinensis* was studied and a micropropagation protocol was developed by Four media were tested Murashige and Skoog (MS), Schenk and Hildebrandt (SH), Driver and Kuniyuk (DKW) and Lloyd and McCown (Woody plant medium, WPM) with shoot-tip and one node cutting for the establishment stage. Murashige and Skoog (MS) was found to be the best medium and one node cutting as starting material for establishment from mother plants. Multiple shoots were obtained on MS medium supplemented with1.00 mgl^{-1} 6-benzyladenine (BA) in the combination with1.00 mgl^{-1} Indole-3-acetic acid (IAA). High frequency of rooting was obtained on full MS solid medium supplemented with 7.00 mgl^{-1}Indole-3-butyric acid (IBA) alone. Regenerated plantlets were successfully transferred to pots containing mixture of peat moss, vermiculite and sand at equal volume with 90% survival.

Keywords: *Simmondsia chinensis*, micropropagation, nodal culture, jojoba, growth regulators.

INTRODUCTION

Jojoba (*Pronounced hohoba*) is a name that is becoming increasingly common at present. Jojoba is found in coastal and cismontane Southern California East to Central Arizona and South to Sonora and Baja California (Munz, 1974; Yermanos, 1974). Now it is cultivated commercially in Argentina, Egypt, India, Mexico, Peru, South Africa and the USA (Kumar *et al*., 2013). The jojoba family (Simmondsiaceae) has only one genus, Simmondsia, consists of only one species, jojoba (*Simmondsia chinensis* (Link) Schneider). Earlier, jojoba was considered as an dated member of the Box family (Buxaceae), but now it is regarded as sufficiently distinct to be placed in a separate family (Munz, 1974; Yermanos, 1974).

It is known as coffee berry, wild hazel, pig nut, gray box bush, goat nut, deer nut and coffee bush. Jojoba is a woody evergreen dioecious shrub or small multi stemmed tree that grows to a height of 0.5-1m in the wild, occasionally to 6m tall with taproots to 12m long. Jojoba is a medicinal and oil-yielding, multipurpose species (Kumar *et al*., 2012), its importance is due to its seeds, which store liquid wax, and is widely used as lubricant, in the antibiotic production, anti-inflammatory, hair care and for medical treatments of stores, wounds, colds, cancer, kidney malfunction and skin disorders, cosmetics, pharmaceuticals, plastic and petroleum industries (Jacoboni; Standardi, 1987 and Mills; Benzioni, 1992).

Jojoba is considered one of the most practical solutions for desert plantation in Egypt, heat, drought and salt tolerance, less possibilities for infection, less need for fertilizers and generous financial income, are certainly the most encouraging goals to plant jojoba in Egypt (El Moguy, 2002).

Propagation of the species is mainly through seeds, due to wind pollination. Therefore, there is a high degree of variation in seed yield and oil content. In a heterogeneous population, identification of male and female plants is not possible until the plant flowers after 3-4 years from cultivation (Yermanos, 1979). In addition, ratio of male to female is normally 5:1(Sharma *et al*., 2008).

Micropropagation is an alternative method of vegetative propagation, which is well suited to the multiplication of elite clones, offers many advantages, is not limited by number of selected elite genotype, produces pathogen-free plants, and can provide a commercial production within a limited time frame and space. The techniques can also be used for genetic improvement of the species (Reddy and Chikara, 2010).

Jojoba plants from tissue culture grow more vigorously than both seedling and rooted cutting and are significantly larger after the first year of growth. Thus micropropagation offers opportunities for the production of thousands of elite plants from the selected stock plant (Lee, 1988).

So this study aimed to establish an applicable protocol to *in vitro* propagation of *Simmondsia chinensis.*

MATERIALS AND METHODS

Plant Material

Plant materials were taken from semi-hard wood stems of jojoba (*Simmondsia chinensis*) female adult tree aged 4 years old from El-Sheikh Zuwyed, Research Station, North Sinai, Desert Research Center (DRC). Actively growing shoots (10 cm in length) were moistened and wrapped with wet paper and transferred to the Lab of Tissue Culture Unit at Plant Production Department, Faculty of Environmental Agricultural Sciences El-Arish, Suez Canal University in March to May during 2012-2015.

Explants sterilization

Shoot-tips and one node cutting of jojoba plants (2-3 cm) in length were excised and washed under running tap water for 1 hr agitated in tap water with a few drops of liquid soap. The explants were soaked for 20 minutes in 25% Clorox (containing 5.25% sodium hypochlorite) with addition of two drops of Tween 20 (used to enhance spreading the disinfectant by reducing the surface tension on plant materials) followed by

immersion in 70% ethanol alcohol for 30 sec. then washed again with sterilized distilled water for 4times to remove all traces of the disinfection.

Culture Medium

The MS, WPM, SH and DKW media containing macro and micro elements as well as vitamins , according to Murashige and Skoog (1962), Lloyd and McCown (1980), Schenk and Hildebrandt (1972) and Driver and Kuniyuk (1984), respectively were used through this study .The media were supplemented with 100 mgl^{-1}myo-inositol and 3% (w/v) sucrose and 0.7% (w/v) agar. All media were adjusted for pH (to 5.6), using either 0.10 N NaOH or 0.10 N HCL depending upon high or low before gelling with 8.00 gl^{-1}agar in all stages. All media were dispensed into each glass tube (25x150 mm) or jar (330ml). The culture tubes and jars were autoclaved at 1.06 kg $cm^{-2}$and 121^{o}C for 20 min.

Establishment stage:

To examine the best medium and explant for establishment of Jojoba, shoot tip and one node cutting were cultured on four different media MS, SH, DKW and WPM.

Scores were given for necrosis estimated as the degree of dead tissues or parts and browning termed as all medium darkened=5, the most of explants dead and the most medium darkened=4, medium=3, less than medium=2, while the healthy and no browning=1. Also explant development was measured as any changed occurred in the explant. Greening (defined as the degree of keeping the original color of the explant and the green color of the leaves. All these data were calculated visually according to Pottino (1981).

Shoot Multiplication

Nodal segments (2-3 cm long) which obtained from establishment stage containing two axillary buds were cut and cultured on MS medium containing BA at 0.00, 0.50, 1.00,1.5 and 2.00 $mgl^{-1}$to detect the best concentration, after that the explants were cultured at BA in combination with Indole-3-acetic acid (IAA) at 1.00, 3.00 and 5.00 mgl^{-1}. Explants were sub-cultured every six weeks. After six weeks of subculture, data were recorded on number of shoots, shoot length and number of leaves.

Rooting stage

For rooting, individual shoots of 3-4 cm long which obtained from multiplication stage were excised from the proliferated shoots and cultured on full and half strengths MS of basal medium supplemented either with 1.00, 3.00, 5.00, 7.00 and 9.00 mgl^{-1} indole-3-butyric acid (IBA) or 1.00, 3.00, 5.00, 7.00 and 9.00 mgl^{-1}(NAA) to determined which the best media strength and type of auxin maximized the highest percentage of roots. Also, isolated shoots were inoculated aseptically on full MS medium with3.00 and 7.00 mgl^{-1} IBA in solid, semi-solid and liquid media. Shoots were cultured on full MS solid media with different concentrations of IBA (1.00, 3.00, 5.00, 7.00 and 9.00 mgl^{-1}) with 2.5gl^{-1} AC. Shoots were cultured on these media for six weeks, and the data were recorded as number of main roots, number of lateral roots, root length and plant length

Acclimatization of plantlets

Well rooted plantlets of Jojoba were subjected to the *in vitro* treatments. The selected plantlets were taken away from the tubes. The roots of the chosen plantlets were washed thoroughly with running water to get rid of residues. The roots were then washed with a sterilized distilled water and planted in black polyethylene pots (8cm in diameter filled with 1:1:1 (v/v/v) sand, peat-moss and perlite), then covered with white transparent bags having small holes made after one week and then widening these holes each week gradually and continuously for four weeks until the plantlets become suitable for transferring to the bigger pots of 30cm diameter where this process named recycling when plantlets produced new leaves they were transferred from greenhouse eventually to field conditions .

Statistical analysis

Experiments were set up in a randomized complete block design (RCBD). There were four replicates and each replicate contained 4 explants for each treatment Data were tested using the analysis of variance (ANOVA) by the General Linear Models (GL Ms) procedures using SAS computer program (SAS, 2004) where a significant difference was observed for the measured value, means were separated using Duncan's multiple range test (DMRT) (Duncan, 1999) at the 5% level.

RESULTS AND DISCUSSION

Establishment stage

Effect of medium and explant type

Regarding the effect of interaction between medium type and explant type it is noticed from Table 1 that the culture of one node cutting on MS medium induced significant reduction in necrosis and browning while increased both explants development and greening parameters. The superiority of one node cutting with MS media was obtained by Bashir *et al.* (2007), Singh *et al.* (2008), Mohasseb *et al*. (2009) and Llorente and Apóstolo (2013) for *Simmondsia chinensis*.

However, Roussos *et al*. (1999) reported that explants were grown in the basal medium of DKW. On the other hand, Chaturvedi and Sharma (1989) observed that the modified (SH) medium achieved the best shoots parameters of jojoba plants

Shoot Multiplication

Effects of various concentrations from BA (0.00, 0.50, 1.00, 1.5 and 2.00 mgl^{-1}) on multiplication of shoots is presented in Table 2. The best number of shoots (1.75), shoot length (4.18) and number of leaves (8.95) of *S. chinensis* were obtained with1.00 mgl^{-1} BA. These results indicated that BA plays a key role in shoot proliferation of *S. chinensis*. The highest number of shoots (2.78) was belonged to the combination between 1 mgl^{-1} BA and 1 mgl^{-1} IAA, while the lowest shoot number (1.00) was belonged to 1 mgl^{-1} BA with 5 mgl^{-1} IAA. These results are in agreement with the findings of Hassan (2003).

Table (1): Effect of medium and explant type on explant development of jojoba plants.

Medium & Explant type		Necrosis	Browning	Explant development	Greening
Murashige and Skoog	**Shoot tip**	2.92^{ab}	1.89^{c}	3.14^{b}	2.83^{b}
	One-node cutting	1.11^{d}	1.56^{d}	4.10^{a}	4.78^{a}
Schenk and Hildebrandt	**Shoot tip**	2.44^{b}	3.10^{a}	1.94^{d}	1.36^{d}
	One-node cutting	2. 77^{ab}	2.33^{b}	2.53^{c}	2.73^{b}
Woody plant medium	**Shoot tip**	3.78 ^{a}	3.22^{a}	2.44^{c}	2.77^{b}
	One-node cutting	3.74 ^{a}	3.30^{a}	2.53^{c}	1.11^{d}
Driver and Kuniyuk	**Shoot tip**	2.78^{ab}	2.12^{b}	1.99^{d}	1.14^{d}
	One-node cutting	2.44 ^{b}	2.20^{b}	1.98^{d}	1.22^{d}

Means followed by the same letters within each column are not significantly different at 0.05 level of probability according to Duncan's multiple range test

Table (2): Effect of BA concentrations on number of shoots, leaves and leaves/shoot and shoot length of jojoba plants.

BA conc.(mgl^{-1})	No. of shoots	No. of leaves	No. of leaves⁄shoot	Shoot/length (cm)
00.0	1.21^{b}	3.00^{b}	1.10^{b}	1.60^{b}
0.50	1.00^{b}	4.50^{b}	1.00^{b}	1.50^{b}
1.00	1.75^{a}	8.95^{a}	2.54^{a}	4.18^{a}
1.50	1.12^{b}	5.00^{b}	1.75^{b}	2.25^{b}
2.00	1.15^{b}	4.33^{b}	1.67^{b}	1.67^{b}

Means followed by the same letters within each column are not significantly different at 0.05 level of probability according to Duncan's multiple range test.

Table (3): Effect of IAA concentrations at a concentration 1mgL^{-1}of BA on shoot length, number of shoot; leaves and leaves/shoot of jojoba plants.

IAA Conc. (mgl^{-1})	Shoot length (cm)	No. of shoot	No. of leaves	No. of leaves⁄shoot
0.00	2.63^{b}	1.69^{b}	2.76^{b}	1.17b^{c}
1.00	4.50^{a}	2.78^{a}	4.89^{a}	3.11^{a}
3.00	3.00^{b}	1.37^{b}	2.60^{b}	2.00^{ab}
5.00	2.60^{b}	1.00^{c}	2.25^{b}	1.00^{c}

Means followed by the same letters within each column are not significantly different at 0.05 level of probability according to Duncan's multiple range test.

Table (4): Effect of medium strength and type of auxin on main roots, lateral roots and leaves/plantlet number, root and plant length of jojoba plants.

Medium strength	Type of auxin	Auxin conc. (mgl^{-1})	No. of main roots	No. of lateral roots	Root length (cm)	plant length (cm)	No. leaves/plantlet
Full	IBA	1	1.00^{c}	1.33^{d}	1.00^{e}	3.57^{c}	5.33^{e}
		3	3.33^{b}	3.35^{c}	6.50^{b}	5.17^{b}	16.00^{b}
		5	1.00^{c}	1.00^{d}	1.00^{e}	3.07^{cd}	7.33^{d}
		7	8.00^{a}	6.33^{a}	10.50^{a}	14.50^{a}	22.00^{a}
		9	1.00^{c}	1.33^{d}	1.00^{e}	2.67^{cde}	6.67^{de}
	NAA	1	1.00^{c}	1.33^{d}	1.00^{e}	1.57^{ef}	1.67^{f}
		3	2.00^{c}	2.00^{d}	2.33^{d}	1.17^{f}	2.33^{f}
		5	1.00^{c}	1.00^{d}	1.00^{e}	1.40^{f}	1.33^{c}
		7	1.67^{c}	2.00^{d}	1.50d^{e}	1.83^{ef}	2.33^{f}
		9	1.00^{c}	1.33^{d}	1.00^{e}	2.67^{cde}	1.33^{c}
Half	IBA	1	1.00^{c}	1.00^{d}	1.00^{e}	1.90^{def}	2.00^{f}
		3	3.00^{b}	5.33^{b}	4.67^{c}	4.80b	9.67^{c}
		5	1.00^{c}	1.00^{d}	1.00^{e}	1.73^{ef}	1.33^{c}
		7	1.00^{c}	1.33^{d}	1.00^{e}	1.37^{f}	1.67^{f}
		9	1.00^{c}	1.33^{d}	1.00^{e}	1.50^{ef}	1.67^{f}
	NAA	1	1.00^{c}	1.00^{d}	1.00^{e}	1.90^{def}	2.00^{f}
		3	1.67^{c}	1.67^{d}	2.33^{d}	1.43^{ef}	1.33^{c}
		5	1.00^{c}	1.00^{d}	1.00^{e}	1.23^{f}	1.67^{f}
		7	1.00^{c}	1.33^{d}	1.00^{e}	1.37^{f}	1.67^{f}
		9	1.00^{c}	1.33^{d}	1.00^{e}	1.50^{ef}	1.67^{f}

Means followed by the same letters within each column are not significantly different at 0.05 level of probability according to Duncan's multiple range test.

Table (5): Effect of type of medium state and different concentrations of IBA on jojoba plant.

Medium State	IBA Conc. mgl^{-1}	Plant Length (cm)	Leaves\ plantlet	No. of main roots
Solid	3.00	5.43^{a}	4.67^{ab}	2.00^{b}
	7.00	5.50^{a}	5.33^{a}	5.00^{a}
Semi solid	3.00	3.83^{b}	3.00^{b}	0.00^{c}
	7.00	5.00^{a}	4.00^{ab}	0.00^{c}
Liquid	3.00	2.33^{c}	0.32^{c}	0.00^{c}
	7.00	2.67^{c}	2.67^{b}	0.00^{c}

Means followed by the same letter within each column are not significantly different at 0.05 level of probability according to Duncan's multiple range test.

Rooting stage

Interaction effect of MS medium strength and type of auxin on rooting

About 3-4 cm long shoots were used for rooting experiment. The rooting response was different according to IBA or NAA as well as the strength of basal salt medium as shown in Table (3). The results indicated that the full MS strength supplemented with 7mgl^{-1} IBA significantly surpassed NAA in increasing number of main roots (8.00), number of lateral roots (6.33), root length (10.50) and plant length (14.50).

Effect of different medium state with full medium strength on rooting traits

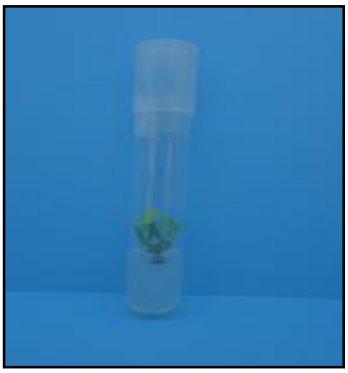
The best medium state was the solid medium with full strength supplemented with 7mg^{-1} IBA which gave the maximum number of main roots (5.00) and plant length (5.50) as show in Table 4. The similar results were found by Chaturvedi and Sharma (1989).who found that about 90% of the isolated shoots of *S. chinensis* rooted in solid medium supplemented with 7 mgl^{-1} IBA, 1 mgl^{-1} NAA and 1 mgl^{-1}caffeic acid per liters. On the other hand, Kacker *et al*. (1993) found that incubated the micropropagated shoots of *S. chinensis* in a liquid half strength MS medium containing 10 mgl^{-1} NAA for 72hr.for early root initiation on subsequent medium and then transferred to half strength MS rooting medium containing (2.5gl^{-1}) AC.

Acclimatization

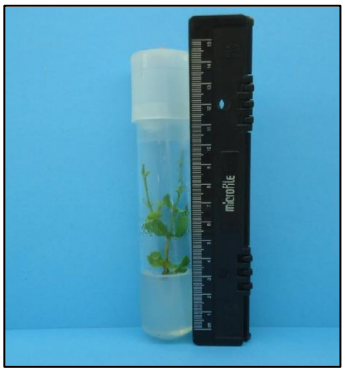
Plantlets regenerated *in vitro* with well-developed root system were transferred to plastic pots containing mixture of peat moss, vermiculite and sand (1:1:1v/v/v) under controlled growth conditions. To maintain high relative humidity, pots were placed in plastic bags. Relative humidity was reduced gradually and complete removal of plastic bags took place after two weeks of placement. The pots with the plantlets were kept in a greenhouse for three weeks for acclimatization. Normal growth of potted plants was visible at 10-15 days after transfer to field conditions. On the other hand, Abass (2010) recorded that rooted shoots were acclimatized and successfully transferred to soil (peat: sand 1:2 v/v) with 60% survival of plants.

CONCLUSIONS

The protocol defined in this study as outlined below and is demonstrated in Fig.1, was found to be efficient and can be utilized for cloning of selected plants of jojoba. Firstly, establishment of *in vitro* shoots from nodal explants on MS medium. Then multiply, the shoots on MS medium +1.00 mgl^{-1} BA+1.00 mgl^{-1}IAA. Moreover, rooting the shoots on full MS solid +7.00 mgl^{-1}IBA alone. Finally hardening the rooted shoots in a greenhouse in pots containing mixture of peat moss, vermiculite and sand (1:1:1v/v/v). The developed protocol can be used to produce uniform and desirable plants for cultivation of *S. chinensis*. It also offers potential system that should be used for improvement, conservation and mass propagation of *S. chinensis*



(1)



(2)

(3)

(4)

Figure (1): Micropropagation of *S. chinensis* from mature plant. (1) *In vitro* shoot establishment from nodal explants on MS medium (2) Multiplication of shoots on MS medium+1.00 mgl^{-1} BA+1.00mgl^{-1} IAA (3) Development of roots on MS solid +7.00 mgl^{-1}IBA (4) Plant raised after transplantation to potting mixture.

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أجريت هذه التجربة بمعمل زراعة الأنسجة النباتية بقسم الانتاج النباتي – كلية العلوم الزراعية البيئية – جامعة قناة السويس خلال الفترة من ٢٠١٢ – ٢٠١٥ بهدف الإكثار الدقيق لنبات الجوجوبا. استخدمت اربعة أنواع من البيئات هي بيئات موارشيج وسكوج (MS) وشنك وهلدبراندت (SH) ودرافير كيونيوك (DKW), ليلويد وماك كوين و(WPM)مع كل من القمة النامية والساق البرعمية في مرحلة التأسيس. وجد أن بيئة موراشيج وسكوج مع الساق البرعمية نتج عنها افضل تطور في الجزء النباتي والمحافظة علي النسيج، كذلك قللت من موت الانسجة النباتية. لإحداث التضاعف معمليا وجد أن تركيز١.٠ ميللجرام/ لتر من البنزيل ادينين مع تركيز١.٠ ميللجرام/ لتر من اندول حمض الخليك مضافان الي بيئة موراشيج وسكوج اعطي اعلي نسبة تضاعف. ادي اضافة تركيز٧.٠ ميللجرام/ لتر من اندول حمض البيوترك منفردا الى القوة الكاملة من بيئة موراشيج وسكوج الصلبة الي زيادة تكوين الجذور. تم أقلمة البادارات بعناية علي بيئة مكونة من البيتموس والفيرميكيوليت والرمل بنسبة ١:١:١ حيث نسبة النجاح ٩٠%.