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Effect of Explant Type and Sequential Subcultures on *In Vitro* Multiple Shoots Formation of Jojoba

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ABSTRACT

Jojoba or Hohoba (*Simmondsia chinensis* (Link) Schneider) is an economically important plant in arid and semi-arid areas. Micropropagation is a highly recommended strategy for obtaining Jojoba elite clones. The present investigation aimed to study the effect of explant type and sequential subcultures on the *in vitro* multiple shoots formation of Jojoba. Four explants form female mature plants were used for the *in vitro* establishment of Jojoba; shoot tips and terminal, sub-terminal and basal stem node segments. It was found that Murashige and Skoog (MS) medium supplemented with 6-benzylaminopurine (BA) at 1.25 mg/l could be a promising treatment for the *in vitro* establishment of all explant types. On the other hand, BA at 2 mg/l is the most promising concentration for shoot multiplication, which gave higher values of mean number of axillary shoots during all successive subcultures than the other treatments. It was also found that the mean number of axillary shoots increased till the 4th subculture and then it decreased in the 5th and 6th subcultures. By comparing the different explant types of Jojoba, shoot tips recorded the highest mean number and length of axillary shoots.

KEYWORDS: *Simmondsia chinensis*, shoot tips, terminal, sub-terminal, basal stem node segments, *in vitro* establishment, multiplication.

Abbreviations: BA, 6-benzylaminopurine; **IBA**, indole-3-butyric acid; **2iP**, N6-(2-isopentenyl) adenine; **Kn,** kintetin; **MS**, Murashige and Skoog

INTRODUCTION

Jojoba or Hohoba (*Simmondsia chinensis*), belongs to family Simmondsiaceae, is a dioecious evergreen perennial desert shrub. It is an economically important plant with natural lifespan of up to 150 years. Jojoba is native to Sonoran Desert of Arizona, California and New Mexico. It starts fruiting from the fourth year of plantation. Female flowers generally bloom in the month of December-January and seeds mature by the last week of May or first week of June. It has an immense economic potential and can be grown on wasteland/coastal sand dunes. Intense interest has developed on its cultivation due to high percentage of seed oil and potential application of oil in cosmetic, petroleum, pharmaceutical and plastic industries (Jacoboni and Standarti, 1987).

Propagation of the species is mainly though seeds, due to wind pollination, therefore, there is a high degree of variation in seed yield and oil content. In a heterogonous population, identification of male and female plants is not possible until the plant flowers. Vegetative propagation could be the alternative for the generation of desired sex specific clones, but it was reported to be difficult (Yermanos, 1979). When the traditional methods are unable to meet the demand for propagation material, micropropagtion can produce millions of uniformly flowering and yielding plants. Therefore, micropropagation of Jojoba offers a promising mean of mass production, pathogen–free superior clones for commercial plantation and production of desired sex–specific clone for its commercial cultivation.

Most of the previous studies on the micropropagation of Jojoba used nodal stem segments as explants source regardless to the position of the nodes on the stem (Jauhar, 1983; Kacker *et al.*, 1993; Gabr, 1993; Agrawal *et al.*, 2002; Tyagi and Prakash, 2004; Bashir *et al.*, 2007; Singh *et al.*, 2008; Mohasseb *et al*., 2009; Llorente and Apóstolo, 2013). Shoot tips were not used previously as source of explants. Therefore, the aim of this study was the use of shoot tips and nodal segments from three different positions on the stem (terminal, sub-terminal and basal stem node segments), and comparing their effect on the *in vitro* establishment and multiple shoots formation of Jojoba to identify the best

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type of Jojoba explant should be used for micropropagation. Also, the influence of six sequential subcultures on the multiplication of shoots was investigated.

MATERIALS AND METHODS

The present study was conducted in Plant Tissue Culture Unit, Plant Genetic Resources Department, Desert Research Center, Cairo, Egypt. Four different Jojoba explants were used to test their ability to be established *in vitro* and six sequential subcultures for multiplication of shoots were carried out.

Explants collection and sterilization

Explants of Jojoba were obtained from mature female shrubs grown in Egyptian Natural Oil Company, El-Manaif, Ismailia and the farm of Faculty of Agriculture, Al-Azhar University. Actively growing shoots (10 cm in length) were collected, moistened and wrapped in wet papers. Explants were divided into shoot tips and stem node segments, which also were divided into 3 parts; terminal, sub-terminal and basal stem node segments. All explants were thoroughly washed with soap under running tap water, for 2-3 h, to remove all the remaining detergent. Surface sterilization was carried out under complete aseptic conditions in the Laminar Air Flow Hood by giving the explants a quick (10 s) rinse in 95% ethyl alcohol and then they were rinsed 3 times with sterile distilled water. Afterwards, explants were immersed in sterile distilled water with 2 drops of Dettol for 10 min, then rinsed 3 times with sterile distilled water. After that, explants were immersed into 0.42% NaOCl solution for 15 min for shoot tips and 1.84% of NaOCl solution for 20 min for the stem node segments. Finally, they were rinsed 3 times with sterile distilled water.

Culture medium and growth conditions

Explants were slightly trimmed at the cutting ends to expose fresh tissue before planting them on Murashige and Skoog (MS) medium (Murashige and Skoog, 1962). The MS medium was supplemented with 3% sucrose, 100 mg/l myo-inositol and was gelled with 0.3% phytagel. Suitable plant growth regulators (PGRs) were used independently or in combinations at different concentrations. After pH adjustment to 5.7-5.8, 15 ml volumes of media were dispensed into 25×150 mm culture tubes or 50 ml volumes into large jars (300 ml). The culture tubes and jars were covered with polypropylene caps and autoclaved at 121°C at a pressured of 1.1 Kg/cm² for 15 min. The sterilized explants were cultured on the prepared media under complete aseptic conditions in the Laminar Air Flow Hood.

All the cultures were maintained in a temperature of 26±2°C under a photoperiod of 16 h with a light intensity of 1500 lux, provided by cool white light fluorescent tubes and 60-65% relative humidity.

In vitro establishment

Establishment MS medium was supplemented with 3% sucrose, 100 mg/l myo-inositol and 0.3% phytagel and different concentrations of BA (0.25, 0.5, 1.25, 1.5, 2.25, 2.5 and 5 mg/l) or 2iP (1, 2 and 2.5 mg/l) and BA (1.25, 2.5 and 5 mg/l) in combination with 2.5 mg/l 2iP, in addition to the control medium (free from PGRs). Percentage of growth induction (%), mean number of axillary shoots/explant and mean length of axillary shoots (cm) were recorded after 8 weeks of culture.

In vitro multiple shoots production

Shoots raised from the establishment stage were subjected to be multiplied on MS medium supplemented with 3% sucrose, 100 mg/l myo-inositol and 0.3% phytagel, in addition to BA (0.5, 1, 2 and 3 mg/l) individually or in combination with 2iP at 1 and 1.5 mg/l, and 2iP (0.5, 1, 2 and 3 mg/l) individually. Explants were also cultured on MS medium supplemented with different concentrations of Kn (1, 2, 3 and 4 mg/l) and TDZ (0.25, 0.5, 1 and 2 mg/l). Percentage of growth induction (%), mean number of axillary shoots/explant and mean length of axillary shoots (cm) were recorded after 8 weeks of culture.

For further multiplication, the explants were subcultured six times on the best multiplication medium every 8 weeks.

Experimental design and Statistical analysis

All experiments were subjected to completely randomized design. Each treatment consisted of at least 10 replicates. Variance analysis ANOVA was done using Costat software program for statistical analysis. The differences among means for all treatments were tested for significance at 5% level by

using new multiple range tests of Duncan (1955) as modified by Snedecor and Cochan (1990). Means followed by the same letter are not significantly different at $p \leq 0.05$.

RESULTS AND DISCUSSION

Growth hormones regulate various physiological and morphological processes in plants and are also known as PGRs or phytohormones (Srivastava, 2002). PGRs are synthesized by plants; therefore many plant species can grow successfully without external medium supplements (Bhavisha and Jasrai, 2003; Baksha *et al.*, 2005). Hormones can also be added into cultures to improve plant growth and to enhance metabolite synthesis (Bhojwani and Razdan, 1996).

1. *In vitro* establishment of Jojoba

1.1. Establishment of shoot tips

The *in vitro* establishment of Jojoba shoot tips is presented in Table 1. Data show the effect of different concentrations of each of BA and 2iP, individually or in combination, in addition to the control medium (free from PGRs) on the establishment of Jojoba shoot tips.

The percentage of growth induction was insignificantly different by using all tested treatments, and reached 100% on MS medium containing 1.25 and 2.25 mg/l BA, individually, in addition to both 1.25 and 2.5 mg/l BA in combination with 2.5 mg/l 2iP.

Table 1. *In vitro* establishment of shoot tips of Jojoba cultured on MS medium supplemented with BA and/or 2iP after 8 weeks of culture.

Cytokinins conc. (mg/l)		% of growth induction	Mean number of axillary shoots/explant	Mean length of axillary shoots (cm)
BA	**2iP**			
0.00	**0.00**	80^{a}	1.2^{bc}	0.580^{c}
0.25	**0.00**	80^{a}	1.2^{bc}	0.790^{bc}
0.50	**0.00**	70^{a}	1.2^{bc}	0.713^{bc}
1.25	**0.00**	100^{a}	2.5^{a}	1.605^{ab}
1.50	**0.00**	60^{a}	1.4^{bc}	1.150^{abc}
2.25	**0.00**	100^{a}	2.8^{a}	0.585^{c}
2.50	**0.00**	90^{a}	1.3^{bc}	0.935^{abc}
5.00	**0.00**	80^{a}	1.3^{bc}	0.730^{bc}
0.00	**1.00**	90^{a}	2.1^{ab}	1.013^{abc}
0.00	**2.00**	80^{a}	1.3^{bc}	1.116^{abc}
0.00	**2.50**	60^{a}	0.7^{c}	1.150^{abc}
1.25	**2.50**	100^{a}	1.4^{bc}	1.875^{a}
2.50	**2.50**	100^{a}	2.3^{a}	1.108^{abc}
5.00	**2.50**	80^{a}	0.9^{c}	1.299^{abc}

Means followed by the same letter within a column are not significantly different at $P \leq 0.05$.

Results show that the concentration of 2.25 mg/l BA gave the highest value for mean number of axillary shoots/explant (2.8 shoots) followed by 1.25 mg/l BA and 2.5 mg/l BA + 2.5 mg/l 2iP, which recorded 2.5 and 2.3 mean number of axillary shoots, respectively. These three treatments were all insignificantly different for their effect on the number of produced axillary shoots. By comparing MS medium containing different concentrations of 2iP individually, it could be noticed that the mean numbers of axillary shoots were decreased while the mean lengths of axillary shoots were high. Using 2iP individually at a high concentration (2.5 mg/l) decreased the mean number of axillary shoots/explant to 0.7 shoots.

With respect to the mean length of axillary shoots; increasing the concentration of 2iP insignificantly increased the mean length of axillary shoots. However, the combination of 1.25 mg/l BA + 2.5 mg/l 2iP recorded the highest mean length of axillary shoots (1.875 cm). While, BA individually gave the least mean lengths of axillary shoots, except the concentrations of 1.25 and 1.5 mg/l BA. In contrast, using BA in combination with 2iP showed higher mean lengths of axillary shoots.

It could be concluded from Table 1 that MS medium containing 1.25 mg/l BA was the most suitable medium for the establishment of Jojoba shoot tips (Figure 1A), which gave the highest

percentage of growth induction (100%) and mean number (2.5 shoots) and length (1.605 cm) of axillary shoots/explant. Addition of 2iP (2.5 mg/l) to the same concentration of BA gave higher mean length of axillary shoots (1.875 cm) with 100% growth induction, but with lower number of axillary shoots (1.4 shoots). Also, MS medium containing both BA and 2iP at a concentration of 2.5 mg/l gave 100% growth induction with significantly high mean number (2.3 shoots) and length (1.108 cm) of axillary shoots.

1.2. Establishment of terminal stem node segments

Results in Table 2 represent the effect of different concentrations of BA and/or 2iP on the *in vitro* establishment of terminal stem node segments of Jojoba. Data show that the growth induction percentage was insignificantly different among all tested treatments, they were all ranged between 80 and 100%.

Table 2. *In vitro* establishment of terminal stem node segments of Jojoba cultured on MS medium supplemented with BA and/ or 2iP. Results were taken after 8 weeks of culture.

Cytokinins conc. (mg/l)		% of growth induction	Mean number of axillary shoots/explant	Mean length of axillary shoots (cm)
BA	**2iP**			
0.25	**0.00**	80^{a}	1.6^{abcd}	0.560^{d}
0.50	**0.00**	100^{a}	1.9^{abc}	1.275^{ab}
1.25	**0.00**	100^{a}	2.1^{ab}	1.200^{abc}
1.50	**0.00**	100^{a}	1.9^{abc}	1.610^{a}
2.25	**0.00**	100^{a}	2.2^{a}	0.555^{d}
2.50	**0.00**	90^{a}	1.8^{abcd}	1.481^{a}
5.00	**0.00**	100^{a}	1.9^{abc}	1.288^{ab}
0.00	**1.00**	80^{a}	1.6^{abcd}	0.617^{cd}
0.00	**2.00**	80^{a}	1.2^{d}	1.175^{abcd}
0.00	**2.50**	90^{a}	1.5^{bcd}	1.715^{a}
1.25	**2.50**	90^{a}	1.8^{abcd}	1.525^{a}
2.50	**2.50**	100^{a}	2.1^{ab}	1.150^{abcd}
5.00	**2.50**	90^{a}	1.9^{abc}	0.726^{bcd}

Means followed by the same letter within a column are not significantly different at $P \leq 0.05$.

The highest value of mean number of axillary shoots/explant reached 2.2 on MS medium containing 2.25 mg/l BA with mean length of axillary shoots of about 0.555 cm, while 2 mg/l 2iP recorded the lowest value of 1.2 shoots. On the other hand, by increasing the concentration of 2iP, the values of mean length of axillary shoots increased. The highest value of mean length of axillary shoots reached 1.715 cm on MS medium containing 2.5 mg/l 2iP, while the lowest value was recorded at the concentration of 2.25 mg/l BA.

It could be noticed from the data in Table 2 that MS medium containing 0.5, 1.25, 1.5, 2.5 and 5 mg/l BA individually gave 90-100% growth induction with significantly the highest values of both mean number and length of axillary shoots. Also, 1.25 and 2.5 mg/l BA + 2.5 mg/l 2iP gave the same results. Accordingly, for the establishment of terminal stem node segments of Jojoba, economically, 1.25 mg/l BA gave the best results, it gave 100% growth induction and mean number of axillary shoots/explant of 2.1 and mean length of axillary shoots of 1.2 cm, which were significantly higher than the other tested treatments (Figure 1B).

1.3. Establishment of sub-terminal stem node segments

The establishment of sub-terminal stem node segments of Jojoba is presented in Table 3. The growth induction percentage was insignificantly different among all tested treatments except for the control MS medium without cytokinins, which gave the least growth induction percentage (60%). The percentage of growth induction reached 100% at media containing 0.5, 1.25 and 2.25 mg/l BA, in addition to that containing 1.25 and 2.5 mg/l BA + 2.5 mg/l 2iP.

The highest mean number of shoots (2.1 shoots) was recorded at concentration of 2.25 mg/l BA, followed by the concentration of 1.25 mg/l BA, which recorded 2 shoots /explant with mean length of axillary shoots of 0.453 and 1.475, respectively. The control medium gave the lowest values of both number (1 shoot) and length (0.445 cm) of axillary shoots. However, using 2iP individually at 2 mg/l

gave the highest mean length of axillary shoots (2.085 cm), while the mean length of axillary shoots on the other treatments were ranged between to 0.445 to 1.475 cm.

Table 3. *In vitro* establishment of sub-terminal stem node segments of Jojoba cultured on MS medium supplemented with BA and 2iP. Results were taken after 8 weeks of culture.

Cytokinins conc. (mg/l)		% of growth induction	Mean number of axillary shoots/explant	Mean length of axillary shoots (cm)
BA	**2iP**			
0.00	**0.00**	60^{b}	1.0^{e}	0.445^{b}
0.25	**0.00**	70^{ab}	1.4^{abcde}	1.215^{ab}
0.50	**0.00**	100^{a}	1.8^{abcd}	0.945^{b}
1.25	**0.00**	100^{a}	2.0^{ab}	1.475^{ab}
1.50	**0.00**	80^{ab}	1.7^{abcde}	0.990^{b}
2.25	**0.00**	100^{a}	2.1^{a}	0.453^{b}
2.50	**0.00**	70^{ab}	1.3^{abcde}	0.450^{b}
5.00	**0.00**	80^{ab}	1.1^{de}	0.776^{b}
0.00	**1.00**	70^{ab}	1.2^{cde}	1.275^{ab}
0.00	**2.00**	90^{ab}	1.9^{abc}	2.085^{a}
0.00	**2.50**	70^{ab}	1.2^{cde}	0.695^{b}
1.25	**2.50**	100^{a}	1.8^{abcd}	0.810^{b}
2.50	**2.50**	100^{a}	1.9^{abc}	0.952^{b}
5.00	**2.50**	90^{ab}	1.6^{abcde}	0.675^{b}

Means followed by the same letter within a column are not significantly different at $P \leq 0.05$.

From the results, it could be concluded that MS medium containing either 1.25 mg/l BA or 2 mg/l 2iP were both the best among the tested treatments with respect to all growth parameters for the establishment of sub-terminal stem node segments of Jojoba (Figure 1C).

1.4. Establishment of basal stem node segments

Different concentrations of BA and/or 2iP were tested for the establishment of basal stem node segments of Jojoba as presented in Table 4. Results show that the growth induction percentage reached 100% on MS medium supplemented with 2.25 mg/l BA, while the other concentrations ranged between 50% to 90%, and 1 mg/l 2iP recorded the lowest growth induction percentage.

Mean number of axillary shoots insignificantly differed among all tested treatments. The concentration of 2.25 mg/l BA recorded the highest value of mean number of axillary shoots/explant (1.7 shoots), while the concentration of 1 mg/l 2iP gave the lowest value.

On the other hand, the mean length of axillary shoots was the highest on MS medium supplemented with 2 mg/l 2iP, which gave the value of 1.745 cm, while the concentration of 5 mg/l BA gave the lowest value (0.475 cm).

So, for the establishment of basal stem node segments of Jojoba, all tested treatments could be used, but by taking into consideration the economic point of view, it is recommended to use the lower concentrations of cytokinins.

From the previous results it can be concluded that MS medium supplemented with BA at 1.25 mg/l could be a promising treatment for the establishment of shoot tips, terminal, sub-terminal and basal stem node segments of Jojoba, where percentage of growth induction reached its highest values, and the mean number of axillary shoots reached to 2.5 for shoot tips, 2.1 for terminal stem node segments, 2 for sub-terminal stem node segments and 1 for basal stem node segments. In this respect Singh *et al*. (2008) mentioned that bud initiation for male and female genotypes of Jojoba was found to be the best on MS medium supplemented with 1 mg/l BA and 12 mg/l adenine. However, Llorente and Apostolo (2013) initiated single node explants of Jojoba on MS medium supplemented with B5 vitamins, 2.5 mg/l BA, 0.1 mg/l IBA, and 0.35 mg/l GA_3, and can be proliferated on MS medium containing B5 vitamins and 1 mg/l BA.

Table 4. *In vitro* establishment of basal stem node segments of Jojoba cultured on MS medium supplemented with BA and / or 2iP. Results were taken after 8 weeks of culture.

Cytokinins conc. (mg/l)		% of growth induction	Mean number of axillary shoots/explant	Mean length of axillary shoots (cm)
BA	2iP			
0.00	**0.00**	80^{ab}	1.5^{a}	1.245^{ab}
0.25	**0.00**	70^{ab}	1.3^{a}	0.725^{bcd}
0.50	**0.00**	80^{ab}	1.4^{a}	1.130^{bc}
1.25	**0.00**	80^{ab}	1.0^{a}	0.930^{bcd}
1.50	**0.00**	90^{ab}	1.3^{a}	0.535^{cd}
2.25	**0.00**	100^{a}	1.7^{a}	0.670^{bcd}
2.50	**0.00**	80^{ab}	1.4^{a}	0.490^{d}
5.00	**0.00**	90^{ab}	1.5^{a}	0.475^{d}
0.00	**1.00**	50^{b}	0.8^{a}	0.800^{bcd}
0.00	**2.00**	80^{ab}	1.1^{a}	1.745^{a}
0.00	**2.50**	80^{ab}	1.6^{a}	1.250^{ab}
1.25	**2.50**	90^{ab}	1.5^{a}	0.581^{cd}
2.50	**2.50**	90^{ab}	1.4^{a}	0.680^{bcd}
5.00	**2.50**	80^{ab}	1.2^{a}	0.600^{cd}

Means followed by the same letter within a column are not significantly different at $P \leq 0.05$.

BA at 1.25 mg/l also recorded high lengths of axillary shoots for shoot tips (1.605 cm), terminal stem node segments (1.2 cm) and sub-terminal stem node segments (1.475 cm) and basal stem node segments (0.93 cm). These results are supported by Bashir *et al*. (2008), who found that the lowest concentration of BA (1.25 mg/l) was better than the highest concentrations, as explants took minimum days (31.5 days) for bud sprouting from nodal segments of Jojoba, while the highest concentrations (5 mg/l BA) caused delay in bud sprouting up to 47.39 days. The reason of the delay in bud sprouting could be the callus formation in some cultures at the highest concentrations of BA. Also, they found that BA (individually) was found better than Kn (individually) or BA + Kn for *in vitro* shoot initiation of Jojoba. BA enhanced shoot proliferation in Jojoba shoot tip and node culture (Roussos *et al.*, 1999).

By comparing the different explant types of Jojoba, it is noticed from the obtained results that the shoot tips found to record the highest mean number and length of axillary shoots. However, Tyagi and Prakash (2004), Singh *et al.* (2008), Mohasseb *et al*. (2009) and Llorente and Apostolo (2013) used the stem node segments. On the other hand, terminal and sub-terminal stem node segments gave higher mean number and length of axillary shoots/explant than basal stem node segments, which gave the lowest mean number and length of axillary shoots/explant, in addition to higher vitrification.

2. Multiple shoots formation

The results of multiplication of the *in vitro* established Jojoba shoots are presented in Table 5. Different concentrations of BA, 2iP, Kn and TDZ were tested for their influence on the multiplication of Jojoba. Shoots cultured on MS medium supplemented with BA individually at a concentration of 2 mg/l gave the highest mean shoots number/explant (4.7 shoots) with mean length of shoots of 0.622 cm. This result is supported by Botti and Zunino (1988) who found that the best response was obtained when explants of Jojoba were cultured on MS medium supplemented with 2 mg/l BA. Also, Prakash *et al*. (2003) showed that BA individually proved to be the best for differentiation of shoots in both male and female explants of Jojoba. This medium was followed by the combination between BA and 2iP at 2 and 1.5 mg/l, respectively, which gave mean number and length of axillary shoots of 3.6 and 0.713 cm, respectively. Also, MS medium supplemented with 2 mg/l BA in combination with 1 mg/l 2iP gave 3.5 mean number of axillary shoots/explant, with longer shoots (1.616 cm), which was insignificantly different from the mean number of shoots on the previous medium. Gabr (1993) found that the combined treatment of BA and 2iP each at 2.5 mg/l was the most promising treatment for the multiplication of Jojoba. And she mentioned that BA was more effective in stimulation of axillary shoot proliferation.

On the other hand, the shoots cultured on MS medium supplemented with Kn individually didn't show any increase in the mean number of axillary shoots, but significantly gave the highest shoot length. Also, shoots cultured on MS medium supplemented with different concentrations of TDZ showed a

decrease in the mean number of axillary shoots, and by increasing the concentration of TDZ, the mean number of axillary shoots decreased, while the mean length of shoots reached their highest values.

Table 5. *In vitro* multiplication of Jojoba shoots cultured on MS medium supplemented with different concentrations of cytokinins. Results were taken after 8 weeks of culture.

(Cytokinins conc. mg/l)				Mean number of axillary shoots/ explant	Mean length of axillary shoots (cm)
BA	2iP	Kn	TDZ		
0.0	0.0	0.0	0.00	2.1^{def}	1.346^{bcdef}
0.5	0.0	0.0	0.00	1.7^{ef}	1.460^{abcde}
1.0	0.0	0.0	0.00	3.5^{abc}	1.862^{abc}
2.0	0.0	0.0	0.00	4.7^{a}	0.622^{h}
3.0	0.0	0.0	0.00	3.4^{bcd}	1.662^{abcd}
0.0	0.5	0.0	0.00	2.1^{def}	1.850^{abc}
0.0	1.0	0.0	0.00	2.6^{bcdef}	1.260^{cdefg}
0.0	2.0	0.0	0.00	1.3^{ef}	1.060^{defgh}
0.0	3.0	0.0	0.00	1.7^{ef}	0.740^{fgh}
0.5	1.0	0.0	0.00	2.5^{bcdef}	1.386^{bcde}
1.0	1.0	0.0	0.00	2.5^{bcdef}	1.483^{abcde}
2.0	1.0	0.0	0.00	3.5^{abc}	1.616^{abcd}
3.0	1.0	0.0	0.00	2.0^{ef}	1.374^{bcde}
0.5	1.5	0.0	0.00	2.1^{def}	0.924^{efgh}
1.0	1.5	0.0	0.00	2.2^{cdef}	2.084^{a}
2.0	1.5	0.0	0.00	3.6^{ab}	0.713^{gh}
3.0	1.5	0.0	0.00	2.0^{ef}	1.132^{defgh}
0.0	0.0	1.0	0.00	1.7^{ef}	1.945^{ab}
0.0	0.0	2.0	0.00	2.0^{ef}	1.464^{abcde}
0.0	0.0	3.0	0.00	2.1^{def}	2.036^{a}
0.0	0.0	4.0	0.00	2.7^{bcde}	1.892^{abc}
0.0	0.0	0.0	0.25	2.4^{bcdef}	1.440^{abcde}
0.0	0.0	0.0	0.50	1.8^{ef}	1.968^{ab}
0.0	0.0	0.0	1.00	1.7^{ef}	1.852^{abc}
0.0	0.0	0.0	2.00	1.2^{f}	1.060^{defgh}

Means followed by the same letter within a column are not significantly different at $P \leq 0.05$

It can be concluded that the use of BA individually at a concentration of 2 mg/l gave the most promising mean number of axillary shoots (Figure 1E), but by addition of 1 mg/l 2iP, the length of shoots increased with insignificant reduction of their number.

Repeated subculture is usually applied for increasing the shoot bud multiplication rate. Table 6 shows the results of six sequential subcultures of Jojoba shoots cultured on MS medium supplemented with 2 mg/l BA individually or in combination with 1.5 mg/l 2iP, which are the best two media for shoots multiplication. Generally, on both media, the mean number of axillary shoots increased till the 4^{the} subculture and then it decreased in the 5th and 6th subcultures. BA individually at 2 mg/l is the most promising concentration, which gave higher values of mean number of axillary shoots during all sequential subcultures than the other treatment. Decreasing of shoot multiplication is might be due to the endogenous hormonal levels of explant. In general the effect of subculturing on multiplication is varies from species to species (Singh *et al*., 2013).

Table 6. *In vitro* multiplication of Jojoba during six sequential subcultures. Results were taken after each 8 weeks of culture.

Cytokinins conc. (mg/l)		1st subculture		2nd subculture		3rd subculture	
2iP	BA	MLS	MNS	MLS	MNS	MLS	MNS
2.0	0.0	4.2^{cd}	2.21^{ab}	4.9^{c}	1.91^{b}	5.7^{b}	1.29^{b}
2.0	1.5	3.7^{d}	2.41^{a}	4.5^{cd}	2.01^{ab}	5.3^{bc}	1.53^{b}
Cytokinins conc. (mg/l)		**4th subculture**		**5th subculture**		**6th subculture**	
2iP	**BA**	**MLS**	**MNS**	**MLS**	**MNS**	**MLS**	**MNS**
2.0	0.0	6.3^{a}	0.82^{b}	5.2^{bc}	1.51^{b}	4.1^{cd}	2.23^{ab}
2.0	1.5	6.1^{a}	0.91^{b}	4.8^{c}	1.93^{b}	3.8^{d}	2.32^{a}

Means followed by the same letter within a column are not significantly different at $P \leq 0.05$
MNS= mean number of axillary shoots/explant
MLS= mean length of axillary shoots/explant

In conclusion, this study proved that shoot tips were the best Jojoba explants for micropropagation, it gave the best results followed by the terminal, sub-terminal and basal stem node segments, respectively. Four successive subcultures are recommended for the multiple shoots formation of Jojobe, since the mean number of axillary shoots increased till the 4the subculture and then it decreased in the 5th and 6th subcultures.

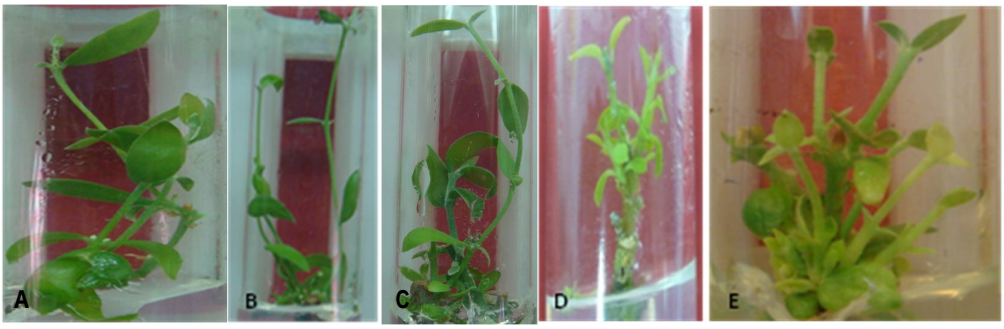

Figure 1. *In vitro* establishment and multiple shoots formation of Jojoba from different types of explants.

A. Establishment of shoot tips of Jojoba on MS medium supplemented with 1.25 mg/l BA.
B. Establishment of terminal stem node segments of Jojoba cultured on MS medium supplemented with 1.25 mg/l BA.
C. Establishment of sub-terminal stem node segments of Jojoba on MS medium supplemented with 2.25 mg/l BA.
D. Establishment of basal stem node segments of Jojoba on MS medium supplemented with 2.25 mg/l BA.
E. Multiple shoots formation of Jojoba on MS medium supplemented with 2 mg/l BA.

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