Tissue culture of *Simmondsia chinensis* (Link) Schneider

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Abstract. Jojoba (*Simmondsia chinensis* (Link) Schneider) a multipurpose and monogeneric dioecious shrub from arid zones, has emerged as a cash crop all over the globe. Its seed propagation poses severe problems due to its male–biased population: the male: female ratio is five to one. To overcome this phenomenon, asexual propagation using vegetative methods such as tissue culture could be used. Investigations were conducted for propagating jojoba plants aseptically *in vitro* staring from nodal segment with two full or half leaves and shoot tip explants. The growth media (MS) were a basal medium additional to Gamborg’s vitamins (B5) and then modified MS medium (MMS) supplemented with different plant growth regulators (PGRs). The results indicated that MS–B5 growth medium was better than MMS medium after 40 and 60 days of culture. Shoot tip explants were superior to nodal segments with two full or half leaves at different concentrations and combinations of PGRs. The highest rooting percentage was recorded in MS/2–B5 + 1 mg/L IBA. This procedure produced an efficient protocol for jojoba tissue culture.

Keyword: Explant, GA3, *In vitro*, Jojoba, PGRs

Introduction

Jojoba (*Simmondsia chinensis* (Link) Schneider) commonly known as jojoba, is a perennial shrub belonging to the Simmondsiaceae family that is native to the Mojave and Sonoran deserts of Mexico, California, and Arizona [SHAH et al., 2010]. Jojoba seeds store lipids in the form of liquid wax that makes up 40–60% of their dry weight. This wax has similar properties to sperm whale oil, and it is used as an industrial lubricant because of its superior lubricating ability and uniform viscosity over a wide range of temperatures [LLORENTE and APOSTOLO, 1998, BOSTAN et al., 2013].

In addition, other uses of jojoba include diverse areas such as pharmaceutical, polishing, and gardening applications.

Jojoba is propagated by sexual and vegetative methods.

In plant populations derived by sexual propagation, it is difficult to determine sex type in early stages of growth i.e., 3–4 years from germination, and plants are genetically variable, which affects growth uniformity, physiological characteristics, yield and early bearing [MOHASSEB et al., 2005, ROUSSOS et al., 1999].

Further, jojoba is biased towards male (5:1; male: female ratio) [AI-OBAIDI et al., 2012].

On the other hand, vegetative propagation methods provide genetically, uniform plant material with early fruiting. Vegetative propagation can be achieved by rooting semi–hardwood cuttings but the maximum number of possible propagules is limited by plant size and time of year. Thus, *in vitro* propagation offers opportunities for the production of thousands of elite plants from the selected stock plant.

Jojoba plants from tissue culture grow more vigorously than both seedlings and rooted cuttings, and are significantly larger after the first year of growth [BIRNBAUM et al., 1985, BUTNARIU and BOSTAN, 2011].

Multiple shoots can be produced *in vitro* and these can be developed into plantlets by regenerating their roots. Thus a single explant source, shoot tip or nodal segment could conceivably provide thousands of new true to type plantlets per year.

A number of workers have described *in vitro* culture of shoot tips [ELHAG et al., 1998, SARDANA and BATRA 1998], nodal

Culture media containing different concentrations of various growth regulators have been used for in vitro shoot initiation of jojoba by a number of researchers.

Mostly cytokinins (BAP [6-Benzyl amino purine], Kinetin and Zeatin) have been used in combination with auxins (NAA [2-(1-Naphthyl)acetic acid], IAA [indole-3-acetic acid], and IBA [Indole-3-butyric acid]) or GA3 [Gibberellic Acid] with varying levels of success.

Botti and Zunino used MS medium containing different concentrations and combinations of BAP, NAA and GA3, the best response was obtained from 2 mg/L BAP in the medium [Botti and Zunino, 1988].

Micropropagated shoots cultured on MS/2 medium containing 3 mg/L IBA gave 31.08% rooting after 70 days [Apostolo et al., 1996, Rashid and Butnariu, 2014a].

The objective of the present study was to propagate *Simmondsia chinensis* in commercial quantities via tissue culture technique, to produce true--to--type plants using direct organogenesis.

**Material and methods**

**Collection of explants**

One and half--year--old greenhouse jojoba plants (*S. chinensis*) propagated by seeds were used as the source of nodal segments with two full and half leaves and shoot tip explants for the present investigation.

Forty--day--old new shoots measuring 15–20 cm in length with four to six nodes were collected in polythene bags and brought to the laboratory for further treatments.

**Preparation and sterilization of explants**

Nodal segments with two full or half leaves and shoot tips 1.0–1.5 cm in length were excised under laminar air flow hood (LAF) and surface sterilized.

Explants were washed under running tap water for ten min (minute) and dipped in a solution of 500 mg/L Tetracycline (Antibiotic) and 4 g/L Yamastin ([Fungicide, Carbendaizm 60 WP] (Yamama Co., Ltd., Jordan) for 15 min followed by three rinses in distilled water. They were treated with 70% ethanol (Changshu Yangyuan Chemical, analytical reagent, China) for 30 sec (second) followed by three washes for one to two min with distilled water.

Under LAF, the shoot tips and nodal segments were immersed in 0.1% HgCl2 (Mercuric chloride) [(w/v) (Qualigens, Mumbai, India)] for five and ten min, respectively.

After treatment both explants were again washed three times for one to two min with sterile distilled water and inoculated onto culture medium (Table 1).

**Culture media and incubation conditions**

The nutrients in growth media consisted of Murashige and Skoog [Murashige and Skoog, 1962, Butnariu et al., 2014] (MS) basal salts additional to Gamborg's [Gamborg's, 1969, Rashid and Butnariu, 2014b] (B5) vitamins and modified MS medium (MMS) containing additional to the basal salts, the following compounds viz., Nicotinic acid, Thiamin HCl, Pyridoxine HCl and Ca pentothenate all at 1.0 mg/L and the rooting medium was MS/2–B5 supplemented with IBA concentrations.

The medium was solidified with 7% agar bacteriological (Himedia, Mumbai, India), and supplemented with 3% sucrose (normal sugar available in the market, Alousra) except in rooting medium where the percentage of sucrose was 2% and different types of PGRs.

The pH of the medium was adjusted to 5.8 after gelling with agar (bacteriological agar) with 1N NaOH or 1N HCl. The growth medium was then dispensed into 25–mL test tubes or onto 250–mL culture bottles and autoclaved for 15 and 20 min at 121°C respectively.

The cultures were incubated at 25±2°C under a 16–hrs photoperiod under cool white fluorescent light (37.5 μmol m–2 s–1).

**Shoot proliferation**

The experiment consisted of 12 treatments i.e., three explants (nodal
segments with two full or half leaves and shoot tips) and different PGRs i.e., BAP at 2.5 mg/L, GA₃ at 0.5 mg/L, IBA, NAA, and IAA all at 0.1 mg/L (Table 1, 2).

The explants were inoculated onto culture bottles with 40 mL or test tubes with 15 mL following surface sterilization.

The data of different shoot proliferation parameters viz., number of days to shoot initiation, number of shoots per explant, number of leaves and nodes per shoot, and shoot length were recorded 40 and 60 days after culture.

Shoot multiplication
The experiment consisted of six treatments i.e., three explants (nodal segments with two full or half leaves and shoot tips) derived from in vitro shoots and MS–B5 supplemented with 30 g sucrose and 1.0 mg/L BAP with or without 0.3 GA₃ mg/L (Table 3, 4).

The data of different parameters of shoot multiplication viz., number of regenerated shoots, number of leaves and nodes per shoot and shoot length were recorded 40 and 60 days after subculture.

Root induction
The experiment consisted of 3 treatments i.e., excised shoots with a minimum stem length of 2–3 cm were transferred to medium with MS/2– B5, and IBA at 1.0, 3.0, and 5.0 mg/L for the randomly selected population (Table 5).

The data of different parameters of rooting viz., percentage of rooting, number of roots per shoot and the root length (cm) were recorded for each shoot 60 days after culture additional to the number of days to root induction according to its emergence.

Experimental design and data analysis
Experiments were conducted in a factorial completely randomized design (FCRD) or completely randomized design (CRD) with three replicates, each with 10 explants per replicate.

Percentage data obtained for various parameters was square root transformed before analyzing data according to Gomez and Gomez, and Sastry [Gomez and Gomez, 1983; Sastry 2007].

ANOVA values were obtained with Opstat1 software (O.P Sheron, Programmer, Computer Section, CCS HAU, Hisar, India) and means were separated with least significant difference (LSD) at P = 0.05.

Results and discussion
Shoot sterilization and proliferation
Almost all produced cultures from nodal segment with two full or half leaves and shoot tip explants treated with above procedure resulted in 85–100% uncontaminated culture (Table 1).

Contamination has often a major hurdle in establishing in vitro cultures with up to 90% losses [Chaturvedi and Sharma 1989].

In our work only less than 15 per cent was lost; this was due to using antibiotic (Tetracycline) with fungicide (Yamastin) and disinfectant chemicals (Ethanol + HgCl₂) additional to the source of explants from greenhouse.

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This result is in line with our previous findings when produced Rangpur lime clean culture on in vitro by the use of fungicides, antibiotics and disinfectant chemicals such as Ethanol and HgCl₂ [EED et al., 2010].

Proliferation of shoots for different parameters of jojoba was studied (Table 1; Figures 1A and 1B).

Effect of growth media and plant growth regulators on establishment of jojoba from nodal segment and shoot tip explants 40 days after culture

<table>
<thead>
<tr>
<th>Plant growth regulators (mg/L)</th>
<th>Clean culture (%) (Mean)*</th>
<th>No. of days to shoot initiation (Mean)*</th>
<th>No. of shoots / explant (Mean)*</th>
<th>No. of leaves / shoot (Mean)*</th>
<th>No. of nodes / shoot (Mean)*</th>
<th>Shoot length (cm) (Mean)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Media</td>
<td>Explant</td>
<td>BAP</td>
<td>GA₃</td>
<td>IBA</td>
<td>NAA</td>
<td>IAA</td>
</tr>
<tr>
<td>MS- B5</td>
<td>N. segment¹</td>
<td>2.5</td>
<td>0.5</td>
<td>0.1</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>N. segment²</td>
<td>2.5</td>
<td>0.5</td>
<td>0.1</td>
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<td>–</td>
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<tr>
<td></td>
<td>Shoot tip</td>
<td>2.5</td>
<td>0.5</td>
<td>0.1</td>
<td>–</td>
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</tr>
<tr>
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<td>0.5</td>
<td>–</td>
<td>0.1</td>
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<td>N. segment²</td>
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<tr>
<td>MMS</td>
<td>N. segment¹</td>
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<td>0.1</td>
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<td>–</td>
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<tr>
<td></td>
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<td>0.5</td>
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<td>Shoot tip</td>
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<td>–</td>
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</tr>
</tbody>
</table>

Similar letters indicate means which are not significantly different (LSD, P = 0.05), comparisons are made in each column, values represent as means. *Nodal segment with two full leaves *Nodal segment with two half leaves *Data inside brackets are square root transformed values

Initiation of shoots after 40 days of inoculation of explants almost was 100% (data not shown). This maximum response for shoot initiation was owing to applying of cytokinins viz., BAP with auxins viz., IBA, NAA and IAA. This might be attributed to cytokinins, which break bud dormancy by activating meristems and causing shoots to proliferate [MURASHIGE, 1974; BUTU et al., 2014].

The outgrowth of axillary buds is in general related with the cytokinin level in the buds. Initiation rates reported here were similar to those reported by Bashir and collab. and Roussos and collab. [BASHIR et al., 2008; ROUSSOS et al., 1999].

Early shoots formation was obtained in MS–B5 medium containing BAP at 2.5 mg/L, GA₃ at 0.5 mg/L and IBA at 0.1 mg/L after 10.33 days with shoot tip explants.

Similarly, same result of shoot formation was recorded in the previous medium with shoot tip explants but with substituting IBA with IAA at 0.1 mg/L.

The longest period for shoot formation (40.00 days) was noticed with nodal segments with two full and half leaves in the medium of MS–B5 supplemented with BAP at 2.5 mg/L, GA₃ at 0.5 mg/L and IBA at 0.1 mg/L.
Almost all initiation media produced shoots early with both explants within 10.33–14.33 days with no significant difference except with the medium already mentioned above.

After 40 days of growth, higher number of shoots and leaves per explant was produced with shoot tip explants in MS–B5 medium supplemented with BAP at 2.5 mg/L, GA3 at 0.5 mg/L and IBA at 0.1 mg/L compared to other explants and media, values were 2.33 shoots and 9.33 leaves respectively.

The greatest number of nodes per shoot was achieved in the MMS medium with nodal segment with two full leaves explants and media.

Shoots were the longest (5 cm) significantly in the MMS medium comprising BAP at 2.5 mg/L, GA3 at 0.5 mg/L and IBA at 0.1 mg/L with shoot tip explants in comparisons with other explants and media whereas the shortest shoots (0.13 cm) were obtained in MS–B5 medium containing BAP at 2.5 mg/L, GA3 at 0.5 mg/L and IBA at 0.1 mg/L with the both type of explants.

However, after 60 days of growth, MS–B5 medium supplemented with BAP at 2.5 mg/L, GA3 at 0.5 mg/L and IBA at 0.1 mg/L with nodal segment explants recorded significantly the greatest number of shoots per explant (2.33 shoots) and with same trend, MS–B5 medium containing BAP at 2.5 mg/L, GA3 at 0.5 mg/L and NAA at 0.1 mg/L with nodal segment with two full leaves explants and MMS medium comprising BAP at 2.5 mg/L, GA3 at 0.5 mg/L and IBA at 0.1 mg/L with nodal segment with two full leaves explants gave same result (2.33 shoots) (Table 2, Figures 1C and 1D).

### Table 2.

Effect of growth media and plant growth regulators on establishment of jojoba from nodal segment and shoot tip explants 60 days after culture

<table>
<thead>
<tr>
<th>Plant growth regulators (mg/L)</th>
<th>No. of shoots/explant (Mean)*</th>
<th>No. of leaves/shoot (Mean)*</th>
<th>No. of nodes/shoot (Mean)*</th>
<th>Shoot length (cm) (Mean)*</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Media</strong></td>
<td><strong>Explant</strong></td>
<td><strong>BAP</strong></td>
<td><strong>GA3</strong></td>
<td><strong>IBA</strong></td>
</tr>
<tr>
<td>MS–B5</td>
<td>N. segment</td>
<td>2.5</td>
<td>0.5</td>
<td>0.1</td>
</tr>
<tr>
<td></td>
<td>N. segment</td>
<td>2.5</td>
<td>0.5</td>
<td>0.1</td>
</tr>
<tr>
<td></td>
<td>Shoot tip</td>
<td>2.5</td>
<td>0.5</td>
<td>0.1</td>
</tr>
<tr>
<td></td>
<td>N. segment</td>
<td>2.5</td>
<td>0.5</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>Shoot tip</td>
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<td>0.5</td>
<td>–</td>
</tr>
<tr>
<td>MS–B5</td>
<td>N. segment</td>
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<td>0.5</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>Shoot tip</td>
<td>2.5</td>
<td>0.5</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>N. segment</td>
<td>2.5</td>
<td>0.5</td>
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</tr>
<tr>
<td></td>
<td>Shoot tip</td>
<td>2.5</td>
<td>0.5</td>
<td>–</td>
</tr>
<tr>
<td>MMS</td>
<td>N. segment</td>
<td>2.5</td>
<td>0.5</td>
<td>0.1</td>
</tr>
<tr>
<td></td>
<td>Shoot tip</td>
<td>2.5</td>
<td>0.5</td>
<td>0.1</td>
</tr>
</tbody>
</table>

Similar letters indicate means which are not significantly different (LSD, P = 0.05); comparisons are made in each column, values represent as means. *Nodal segment with two full leaves. **Nodal segment with half leaves.

Similarly, MS–B5 medium supplemented with BAP at 2.5 mg/L, GA3 at 0.5 mg/L and IBA at 0.1 mg/L with shoot tip explants obtained significantly the greatest number of leaves per shoot (10 leaves) compared to other explants and media.

The highest number of nodes per shoot and the longest shoots were observed with nodal segments with two half leaves in MS–B5 medium supplemented with BAP at 2.5 mg/L, GA3 at 0.5 mg/L and IAA at 0.1 mg/L (3.33 nodes and 8.00 cm respectively) and also in MMS medium supplemented with BAP at 2.5 mg/L, GA3 at 0.5 mg/L and IBA at 0.1 mg/L with shoot tip explants (3.33 nodes and 8.00 cm respectively).

From the mentioned results already, almost the shoot tip explants were more responsive for shoot proliferation in different parameters studied, perhaps due to presence of undeveloped buds.

According to Singh and Singh [SINGH and SINGH, 2005], cytokinins generally act in combination with auxins, cytokinins stimulate cell division even in non–meristematic tissues. In parenchyma, cell division occurs only when both auxins and cytokinins are present.
Furthermore, the ratio of cytokines to auxins controls cell differentiation and when the ratio is in the favour of cytokinins, shoot formation takes place.

**Shoot multiplication**

Table 3 and Figure 2A showed differences in the values among various explants and media after 40 days of multiplication but with no significant difference with respect to the characters of number of shoots per explant and number of leaves and nodes per shoot.

**Table 3.**

<table>
<thead>
<tr>
<th>Media</th>
<th>Explant</th>
<th>Plant growth regulators (mg/L)</th>
<th>No. of shoots / explant (Mean)*</th>
<th>No. of leaves / shoot (Mean)*</th>
<th>No. of nodes / shoot (Mean)*</th>
<th>Shoot length (cm) (Mean)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>MS–B5</td>
<td>N. segment¹</td>
<td>1.0 0 2.00 a</td>
<td>5.66 a</td>
<td>3.66 a</td>
<td>3.66 c</td>
<td></td>
</tr>
<tr>
<td>MS–B5</td>
<td>N. segment²</td>
<td>1.0 0 6.33 a</td>
<td>11.00 a</td>
<td>4.66 a</td>
<td>4.50 bc</td>
<td></td>
</tr>
<tr>
<td>MS–B5</td>
<td>Shoot tip</td>
<td>1.0 0 3.66 a</td>
<td>8.33 a</td>
<td>3.66 a</td>
<td>5.00 ab</td>
<td></td>
</tr>
</tbody>
</table>

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¹Nodalsegment with two full leaves.
²Nodal segment with tow half leaves

The greatest number of shoots per explant and number of leaves and nodes per shoot (6.33–11.00–4.66 respectively) was recorded in MS–B5 with BAP at 1.0 mg/L and without GA₃ with nodal segments with two half leaves.

The highest shoot length was seen with nodal segment explants with the two half leaves in MS–B5 medium containing BAP at 1 mg/L and GA₃ at 0.3 mg/L, the value was 5.83 cm.

**Figure 2. In vitro multiplication and rooting of jojoba.** (A) Shoot multiplication 40 days after subculture in vitro nodal segment explants in MS–B5 medium + 1.0 mg/L BAP; (B) shoot multiplication 60 days after subculture in vitro nodal segment explants in MS–B5 medium + 1.0 mg/L BAP (C–D) Root induction 60 days after subculture shoots in MS/2–B5 + 1.0 mg/L IBA

While after 60 days of growth of explants in the multiplication medium, no significant difference was observed in terms of number of shoots per explant and number of leaves per shoot (Table 4 and Figure 2B).

**Table 4.**

<table>
<thead>
<tr>
<th>Media</th>
<th>Explant</th>
<th>Plant growth regulators (mg/L)</th>
<th>No. of shoots / explant (Mean)*</th>
<th>No. of leaves / shoot (Mean)*</th>
<th>No. of nodes / shoot (Mean)*</th>
<th>Shoot length (cm) (Mean)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>MS–B5</td>
<td>N. segment¹</td>
<td>1.0 0 2.66 a</td>
<td>10.00 a</td>
<td>4.66 b</td>
<td>9.66 a</td>
<td></td>
</tr>
<tr>
<td>MS–B5</td>
<td>N. segment²</td>
<td>1.0 0 6.33 a</td>
<td>32.00 a</td>
<td>3.00 c</td>
<td>7.00 b</td>
<td></td>
</tr>
<tr>
<td>MS–B5</td>
<td>Shoot tip</td>
<td>1.0 0 3.33 a</td>
<td>18.00 a</td>
<td>4.00 bc</td>
<td>6.66 b</td>
<td></td>
</tr>
</tbody>
</table>

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¹Nodalsegment with two full leaves.
²Nodal segment with tow half leaves

50
The highest number of shoots per explant (6.33) and number of leaves per shoot (32.00) were observed in the MS–B5 containing 1.0 mg/L BAP and without GA₃ with nodal segment explants with two half leaves compared to other explants.

Significantly, greatest number of nodes per shoot (8.00) was formed in the MS–B5 containing BAP at 1.0 mg/L and GA₃ at 0.3 mg/L with nodal segment explants with two half leaves in comparison with other explants.

The longest shoots (9.66 cm) were achieved in the MS–B5 medium with 1.0 mg/L BAP and without GA₃ with nodal segment explants with two full leaves followed by the same medium containing BAP at 1.0 mg/L and GA₃ at 0.3 mg/L (9.33 cm) with nodal segment explants with two half leaves with no significant difference between both of them but they differed significantly with the remaining values of shoot length.

**Root induction**

Stem cuttings of these plants have been reported to be difficult to root in vitro as well as in soil [ARAGAO and HOGAN, 1976; CHATURVEDI and SHARMA, 1989; GARCIA BERENGUER, 1992; EED and BURGOYNE, 2014].

Root induction was done successfully in MS/2–B5 medium comprising different concentrations of IBA with selected in vitro shoots (Table 5 and Figures 2C, 2D).

### Table 5.

Effect of different concentrations of IBA on in vitro rooting of jojoba shoots in MS/2–B5 medium

<table>
<thead>
<tr>
<th>Medium</th>
<th>No. of days to root formation (Mean)*</th>
<th>% Rooting (Mean)*</th>
<th>No. of roots/shoot (Mean)*</th>
<th>Root length (cm) (Mean)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>MS/2–B5 + 1mg/L IBA</td>
<td>50 a</td>
<td>33.33 a (5.84)</td>
<td>6.66 a</td>
<td>4.33 a</td>
</tr>
<tr>
<td>MS/2–B5 + 3mg/L IBA</td>
<td>44 a</td>
<td>24.44 a (5.03)</td>
<td>3.66 b</td>
<td>2.66 b</td>
</tr>
<tr>
<td>MS/2–B5 + 5mg/L IBA</td>
<td>45 a</td>
<td>11.10 b (3.44)</td>
<td>2.33 b</td>
<td>1.66 b</td>
</tr>
</tbody>
</table>

* Similar letters indicate means which are not significantly different (LSD, P = 0.05), comparisons are made in each column; values represent as means. 1Data inside brackets are square root transformed values.

Almost rooting of jojoba shoots were formed within 44–50 days with no significant difference among various periods. The minimum concentration of IBA (1.0 mg/L) gave significantly the highest rooting percentage (33.33 %) compared to the other IBA concentrations with less quantity of callus.

This was followed by IBA concentration of 3 mg/L with rooting percentage of 24.44 % then IBA at 5 mg/L with rooting percentage of 11.10 % which was the lowest per cent obtained.

IBA was used for in vitro rooting because it is the most common growth regulator used to stimulate in vivo rooting [LOW and HACKETT, 1981] and the optimum IBA concentration in our experiment was 1 mg/L.

This result also agreed with the findings reported by Llorent and Apostolo [LLORENT and APOSTOLO, 1998].

### Conclusions

An efficient protocol was standardized for micropropagating of jojoba plants. The best growth medium for shoot sterilization and proliferation was MS-B5 medium supplemented with BAP at 2.5 mg/L, GA₃ at 0.5 mg/L and IBA at 0.1 mg/L with shoot tip explants.

The MS/2 medium containing 1 mg/L IBA was ideal for root induction.

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### References


