

## **In Vitro Clonal Propagation of Jojoba (*Simmondsia Chinensis* (Link) Schn.).**

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**Abstract:** *In vitro* clonal propagation of a 5-year-old jojoba tree through axillary shoot proliferation was achieved. Nodal explants collected between March and May gave the best shoot proliferation response (80%) aseptic cultures. MS (major inorganic salts reduced to half strength) medium was required initially for the establishment of nodal segment cultures. Multiple shoot formation was occurred in the cultures of MS+BAP (1 mg /l) + GA<sub>3</sub> (0.5 mg /l). Number of shoots enhanced further on transfer of cultures to MS+BAP (1 mg /l) + CH (500 mg /l). However, both of the media did not support shoot growth and the shoots remained compact and stunted. Therefore, for elongation and recurrent shoot multiplication, full MS+BAP (1 mg /l) + CH (250 mg /l) medium was used where the shoots elongated well, and could be multiplied through single node segment cultures at a rate of 7–8-fold every 5 weeks on the fresh medium of the same composition. This rate of shoot multiplication was maintained for almost 1 year. On the other hand, 1/4 MS+IBA (0.5 μM), the shoots could be readily rooted with a frequency as high as 82%. Successful rooting of jojoba shoots derived from shoot tips and nodal segments were reported on MS medium supplemented with 1.0 mg /l and 2.0 mg /l of AC combined with 5mg/l IBA. Micropropagated plantlets were transplanted in soil with more than 70% survival.

**Key words:** Axillary-shoot-proliferation; Clonal propagation; Jojoba; Nodal segment culture

### **INTRODUCTION**

Jojoba [*Simmondsia chinensis* (Link) Schneider] is a desert shrub which tolerates saline and alkyle soils and drought. The seeds contain a characteristic liquid wax of economic importance in industry (machine lubricant) and medicine (e.g. cosmetics and anticancer compounds, (Fernanda, *et al.*, 2009 and Mills *et al.*, 2004)

A major problem in seeds production is that jojoba is a dioecious plant where its sex is not easily determined prior to flowering (3-4 years from cultivation). Plant regeneration via tissue culture is an important tool in mass propagation, mutant selection and genetic transformation. Tissue culture techniques have been applied only to a limited extent in jojoba. (Roussos, *et al.*, 1999 and Agrawal *et al.*, 2002)

Micropropagation of jojoba offers a promising method for mass production of superior pathogen-free clones for commercial plantations (Mills *et al.*, 1997 and). In the protocol, jojoba sealed tubes were recommended in all four stages of *in vitro* propagation. However, jojoba plantlets produced in tightly closed growth vessels exhibited anatomical, morphological, and physiological abnormalities (Apostolo and Llorente, 2000, Dror, 1981) due to hyperhydricity (Ziv, 1991).

The advantages of using asexual propagules in commercial jojoba plantations are that they provide uniform and predictable plant growth and yield (Lee 1988). Furthermore, jojoba is dioecious and cannot be sexed until flowers appear (usually 2-4 years from seed). Clonal propagation of elite individuals of known sexuality is necessary to ensure that the plants in commercial plots will be productive (Chaturvedi and Sharma 1989). 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 planting (Low and Hackett 1981). Jojoba plants from tissue culture grow more vigorously than both seedlings and rooted cuttings, 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).

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Consequently, several investigators have attempted clonal propagation of jojoba tree (Roussos, *et al.*, 1999; Mills *et al.*, 2009 and Tyagi and Prakash, 2004). Most of these reports deal with juvenile material or material of unspecified age, which is of little significance in clonal propagation of elite trees. Most other reports lack crucial information such as the rate of multiplication in recurrent subcultures. However, the present study, reports a highly reproducible and recurrent method of clonal propagation of a 5-year-old jojoba tree through axillary shoot proliferation.

The promotary effects of AC on morphogenesis may be mainly due to its irreversible adsorption of inhibitory compounds in the culture medium and substantially decreasing the toxic metabolites, phenolic exudation and brown exudate accumulation. The effect of AC on growth regulator uptake is still unclear but some workers believe that AC may gradually release certain adsorbed products, such as nutrients and growth regulators which become available to plants. This review focuses on the various roles of activated charcoal in plant tissue culture and the recent developments in this area (Thomas and Michael, 2007; Thomas, 2008; Sul and Korban, 2005 and Shi *et al.*, 2008).

In fact that, there is no real wax oil industry in Egypt and this due to insufficient plant material for growing in the desert and other place in Egypt. Jojoba is clonally propagated by nodes and the rate of propagation is very limited because the nodes are hard to roots, so that the only solution to solve this problem is through the rapid mass production by tissue culture technique. Jojoba is a tropical and subtropical plant; the main row material is Wax Esters (Jojoba oil) production and has a big market in the entire world.

## MATERIAL AND METHODS

### ***Plant Material and Initiation of Aseptic Cultures:***

For the experiments of the randomly population, explants were taken from semi-hardwood stems of field-grown jojoba adult plants from the Egyptian Natural Oil Co. S.A.E. by Contact: Dr. Nabil Sadek El Mogy, Chairman.

Single node cuttings (2 cm) from a 5-year-old tree, were collected at monthly intervals over three consecutive years to initiate cultures. After removing leaves, the cuttings were thoroughly washed with 1% solution of savlon for 20 min and rinsed twice in sterile distilled water (SDW). All subsequent operations were carried out inside a laminar air-flow cabinet. The clean cuttings were given a quick (30 s) rinse in 70% ethanol, followed by two washings in SDW. These were then surface-sterilized in 0.15% mercuric chloride (HgCl<sub>2</sub>) solution for 13 min and rinsed thrice with SDW. The cuttings were slightly trimmed at both ends to expose fresh tissue before planting them on Murashige and Skoog (MS) medium [1962] containing 3% sucrose and gelled with 0.8% agar.

The MS medium was supplemented with plant growth regulators and other adjuvants. After adjusting the pH to 5.8, 20 ml of medium was dispensed into each 150×25 mm Borosil rimless glass tube. The culture tubes and jars were wrapped and autoclaved at 1.06 kg cm<sup>-2</sup> and 121 °C for 15 min. Thermolabile compound, such as gibberellic acid (GA<sub>3</sub>), was filter-sterilized and added to the autoclaved medium cooled to 50 °C; medium was then dispensed into glass tubes inside a laminar air-flow cabinet. All the cultures were maintained in diffuse light (1000–2000 lx) and 16 h photoperiod at 25±2 °C and 50–60% relative humidity.

In the preliminary experiments, the explants were cultured onto either full-strength Murashige & Skoog (MS) or half-strength MS (1/2 MS, major inorganic salts reduced to half-strength) basal medium. Subsequently, 1/2 MS medium was tested with BAP (1 µM) alone or in combination with GA<sub>3</sub> (0.5 µM). The nodal explants with clusters of shoots produced by 5-week-old primary cultures of nodal segments on 1/2 MS+BAP (1 µM) + GA<sub>3</sub> (0.5 µM) were transferred to 1/2 MS+BAP (1 µM) + 500 mg l<sup>-1</sup> CH (Casein hydrolysate; contains Vitamin B<sub>12</sub>, Pepsin, Trypsin, Papain) for further shoot multiplication.

### ***Multiplication of Shoots:***

Small (0.5 cm) individual shoots from 5-week-old cultures of 1/2 MS+BAP (1 mg l<sup>-1</sup>) + CH (500 mg l<sup>-1</sup>) were excised carefully and transferred to full-strength MS medium supplemented with BAP (1 mg l<sup>-1</sup>) and lower concentration of CH (250 mg l<sup>-1</sup>) for elongation of shoots. Though each axillary's shoot, which had grown fairly long, was cut into single node segments and transferred to fresh medium for further multiplication. The number of propagules obtained at the end of a multiplication cycle was regarded as the rate of shoot multiplication. And other multiplication treatments examined were

Full MS+ 1.5 mg l<sup>-1</sup>BA+ 1.5 mg l<sup>-1</sup>kin  
Full MS+ 2.0 mg l<sup>-1</sup>BA+ 1.5 mg l<sup>-1</sup>kin  
Full MS+ 3.0 mg l<sup>-1</sup>BA+ 1.5 mg l<sup>-1</sup>kin  
Full MS+ 4 mg l<sup>-1</sup>BA+ 1.5 mg l<sup>-1</sup>kin

At least 24-cultures were raised for each treatment and all the experiments were repeated at least three times. Observations on number of cultures showing contamination, bud-break, shoot elongation and rooting were made at weekly intervals. Standard error of the mean was calculated and is indicated by  $\pm$  sign.

#### **Rooting and Adaptations:**

For rooting, terminal 3 cm long portions of elongated shoots with 3–4 nodes were excised and cultured on MS medium supplemented with indole-3-butyric acid (IBA) in the range of 1 to 5 mg l<sup>-1</sup>.

Full MS+ 1.0 mg l<sup>-1</sup>IBA  
Full MS+ 2.0 mg l<sup>-1</sup>IBA  
Full MS+ 3.0 mg l<sup>-1</sup>IBA  
Full MS+ 4.0 mg l<sup>-1</sup>IBA  
Full MS+ 5.0 mg l<sup>-1</sup>IBA

Shoot proliferation was successful at all the concentrations tested, with a maximum number of 15-20 shoots per original explant. When the rooted explants were transferred to the mist system for acclimatization, 70% of them survived and continued to grow after a period of one month.

#### **The Role of Activated Charcoal (AC) in Jojoba:**

Another treatments examined for rooting were MS basal medium with 5mg/l IBA combined with 0.5, 1.0, 2.0 and 3.0 g/l AC respectively to simultaneously promote both elongation and rooting.

#### **Transplantation:**

The rooted plantlets were washed to remove the agar, then transplanted in soilrite (contains peat moss with vermiculite) in hycotrays and placed in a glasshouse at the Tissue Culture Pilot Plant of the Plant Biotechnology Department at National Research Centre. The glasshouse is furnished with facilities to maintain a gradient of humidity by the Fan-and-Pad system and a temperature of 25 $\pm$ 2 °C. Close to the pad, which is continuously drenched with water, the relative humidity (RH) is almost 100% and it gradually decreases towards the other end of the glasshouse. Initially, the plants were placed close to the pad and covered with cling film. Thereafter, the plants were gradually moved away from the pad end. After another 4 weeks the plants were transferred to pots and shifted to a polyhouse (at 25 $\pm$ 2 °C). After 3 months of transplantation, the plants were shifted to a shaded area under natural conditions.

## **RESULTS AND DISCUSSION**

Initiation and *in vitro* propagation of jojoba in general is difficult due to various problems, mainly recalcitrance of the tissue, contamination and field establishment. Joarder *et al.*, (1993) found that the pre-culture of nodal segments, taken from 30-year-old neem tree, on MS basal medium for 2 weeks followed by 4-weeks on MS+BAP (1.5 mg l<sup>-1</sup>) medium was essential/ beneficial for bud-break to occur. Although the paper lacks details regarding the rate of shoot multiplication in subsequent subcultures, initially two shoots developed per node and the number of shoots increased with the increasing number of subcultures and then declined after five to six subcultures

#### **Establishment of Aseptic Nodal Segment Cultures:**

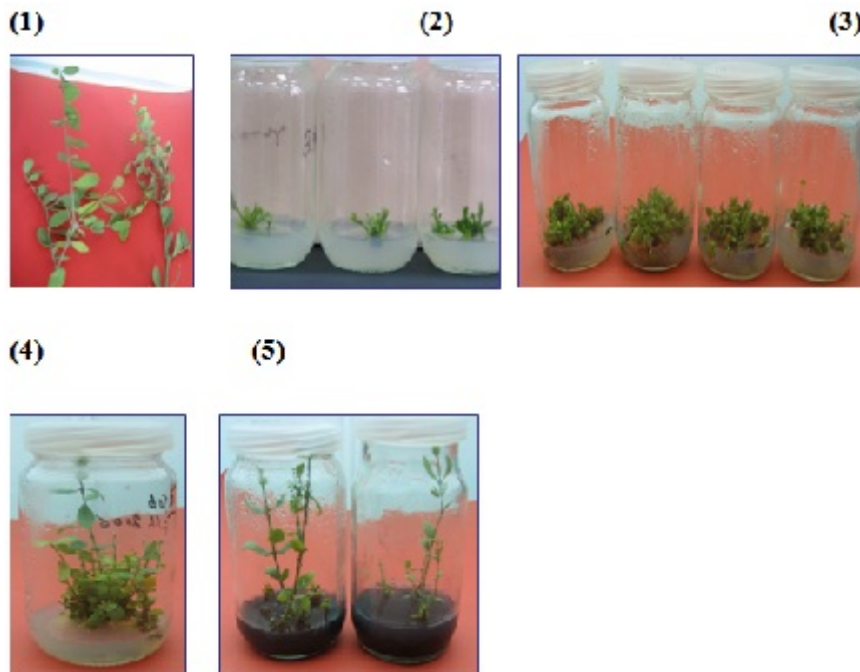
Contamination of jojoba (*Simmondsia chinensis*) nodal explants (Fig. 1), was a major problem during initiation of cultures under *in vitro* conditions. The explants were mainly contaminated by fungus and rarely by bacteria. The rate of contamination or bud-break was highly dependent on the season during which the material was collected. By the sterilization procedure described in materials and methods, the cultures initiated in March–May (during normal flowering in Egypt) showed higher bud-break (80%) and less contamination (20%) than those raised in other time.

Therefore, routinely, the cultures were raised in March–May because of the least contamination and best shoot growth recorded in this season. Similar seasonal effect on culture establishment has been reported for

apple (Hutchinson, 1984), Papaya (Litz and Conover, 1981), sweet gum (Sutter and Barker, 1985) and guava (Amin and Jaiswal, 1987). Sharma *et al.*, (1999) could obtain, after nine steps of rigorous surface sterilization, only 20% aseptic cultures initiated in March–April from 3 to 4-year-old trees of jojoba.

**Elongation and Multiplication of Shoots:**

Axillary shoot proliferation from the nodal explants of jojoba varied considerably at different growth regulator concentrations in the medium, and MS basal medium with half the concentration of major inorganic salts (1/2 MS) showed bud-break in 20% (1.0 nodes/shoot) of the cultures as against 5% (1.0 nodes/shoot) cultures at full concentration. Most of the nodal explants turned brown at (MS) concentration after 2 weeks of incubation; hence for establishing nodal segment cultures MS medium was used initially. Addition of 1 mg /l BAP to MS medium+0.5 mg /l GA<sub>3</sub> (fig. 2)enhanced the frequency of bud-break (50%). However, only a single shoot (3 cm long) developed per node. The shoots were not very healthy; yellowing and abscission of leaves occurred after 4 weeks. Incorporation of kin (1.5 mg /l) to MS+BAP (1.5 and 4.0 mg /l) medium improved the incidence of bud-break and promoted multiple shoot formation (fig. 3). On MS+BAP (3 mg /l) + kin (1.5 mg /l) medium bud-break occurred within 3 weeks and an average of 8-15 small shoots per explant were formed within 4 weeks in 70% cultures. However, the shoots remained very small (<5 mm) even after 5 weeks (Fig. 4).



**Fig. 1:** (1)jojoba male and female branches before starting the tissue culture (2)Five-week-old single node segment cultures on MS+ GA<sub>3</sub> (0.5 mg /l ) + BAP (1.0 mg /l ), showing the initiation of shoot production at the node; the shoots did not elongate. (3) Cultures transferred from 2 to MS+BAP (1.0 mg /l) + kin (1.5 mg /l) + CH (500 mg l<sup>-1</sup>). (4) Some of the shoots have elongated and some new shoots have differentiated after 5 weeks . (5) An excised shoot from B, 5 weeks after transfer to MS+BAP (1 μM) + kin (1.5 μM) + CH (250 mg l<sup>-1</sup>). The shoot has elongated considerably.

In the second passage, the explants with clusters of 3–4 shoots from MS+BAP (3 μM) + kin (1.5 μM) were transferred after 5 weeks to MS+BAP(1μM)+CH (500 mg l<sup>-1</sup>) medium. The number of shoots enhanced further and an average of 7.5 shoots per explant were formed after 5 weeks in 91% cultures (Fig. 4). One of the explants produced as many as 20 shoots. However, most of the shoots remained compact and stunted and did not grow beyond 0.7 cm. Shoot proliferation was associated with the proliferation of callus at the base of the explant only. .

Joshi and Thengane (1996) cultured nodal segments from 2–5-year-old juvenile trees as those from 15–20-year-old mature trees showed negligible bud-break. Sharma *et al.*, (1999) cultured nodal segments from 3, 7-

and 40-year-old trees and found the explants from younger trees to be more responsive. Shoot multiplication at a rate of five-fold in 30 days was achieved, generally, after the fifth passage on MS medium supplemented with 0.25 mg l<sup>-1</sup> each of BAP and IAA and 15 mg l<sup>-1</sup> adenine sulphate. However, in the explants taken from 3-, 7-year-old trees, this rate of shoot multiplication was achieved even by the third subculture. Islam *et al.* (1997) recorded 4.5 shoots per explant up to fourth subculture from nodal segments of 25-year-old tree on MS medium supplemented with 1 mg l<sup>-1</sup> each of BAP and Kn.

MS+BAP (3 mg /l) + kin (1.5 mg /l) proved optimum for shoot multiplication but it did not facilitate shoot growth. Therefore, 0.5 cm long individual shoots were excised carefully after 5 weeks and transferred to MS+BAP (1 mg /l) supplemented with 250 mg l<sup>-1</sup> CH for elongation. On MS+BAP (1 mg /l)+ CH (250 mg l<sup>-1</sup>), the original shoot attained a length of 8.5 cm, with 8 nodes, after 5 weeks in 95% cultures (Fig. 5). Thus, eight-fold shoot multiplication every 5 weeks could be achieved on MS+BAP (1 mg /l) + CH (500 mg l<sup>-1</sup>) by cutting the solitary shoot into single node segments and culturing them on fresh medium. This rate of shoot multiplication was maintained for almost 2 years. More than 30 times the nodal explants were taken and used for shoot multiplication. Since every time the explants were taken from freshly formed in vitro shoots, therefore, we have not observed any significant difference (variation) in the results. This was observed for 2 years and after that all the cultures were given to Tissue Culture Pilot Plant of National Research Centre, Egypt.

Islam *et al.*, (1997) used a medium with a lower level of BAP (0.1 mg l<sup>-1</sup>) for shoot elongation, suggesting that the shoots remained very small on the multiplication medium and required an elongation step to obtain shoots suitable for rooting.

Micropropagation of jojoba offers a promising method for mass production of superior pathogen-free clones for commercial plantations. In the original protocol of (Mills *et al.*, 1997 Mills *et al.*, 2009 and Tyagi and Prakash, 2004) for the micropropagation of jojoba sealed tubes were recommended in all four stages of in vitro propagation. However, jojoba plantlets produced in tightly closed growth vessels exhibited anatomical, morphological, and physiological abnormalities (Apostolo and Llorente, 2000 and Mills *et al.*, 1997) due to hyperhydricity (Ziv, 1991).

#### **Rooting of Shoots:**

Terminal 3 cm long portions of shoots from 5-week-old cultures on MS+BAP (1 mg /l) + CH (250 mg l<sup>-1</sup>) were used for rooting. The remaining portions of the shoots were cut into single node segments and utilized for further multiplication. For rooting, MS was tested at full (MS) strength of the major inorganic salts. All media were supplemented with IBA at 1.0 mg /l to 5.0 mg /l. Rooting was induced directly at the base of the shoot on 1,2,4,5 mg /l IBA (fig. 6) whereas it was preceded by callusing at the base of the shoot on 3 mg /l IBA. On MS+ 5.0 mg /l IBA, which proved to be the best rooting medium, (fig.7) 82% shoots formed an average of 8.4 roots directly from the basal end of the shoot. Even the root length and the number of laterals were maximum on this medium (Fig. 8). On this medium roots appeared after 3 weeks and maximum response was observed after 4 weeks.

Successful rooting of camphor tree (*Cinnamomum camphora*) shoots derived from shoot tips and nodal segments were reported on WPM medium supplemented with AC, IBA and NAA. 100% of the shoots rooted on IBA and 2 g/l AC containing medium (Nirmal Babu *et al.*, 2003).

Most of the investigators could achieve 70–100% rooting of the in vitro regenerated or multiplied shoots of jojoba. However, the optimum conditions described vary considerably. Generally an auxin has been necessary, and MS with silver nitrate has been found to be better than MS alone.

Whereas Joshi and Thengane (1996) and Venkateswarlu and Mukhopadhyay ., (1999) found IAA (2–3 mg l<sup>-1</sup>) to be the best auxin for rooting of neem shoots, Joarder *et al.*, (1993) and Islam *et al.*, (1997) found IBA (0.5 mg l<sup>-1</sup>) to be most effective. Gill *et al.* (1996) and Roy *et al.*, (1996) reported rooting in the combined presence of IBA and IAA. Sharma *et al.*, (1999) found shoots multiplied from a 4-year-old tree to be recalcitrant for rooting. However, in the present study the shoots from a 2-year-old tree could be readily rooted on MS+0.5 mg l<sup>-1</sup> IBA, with a frequency as high as 82%. (Fig 7)

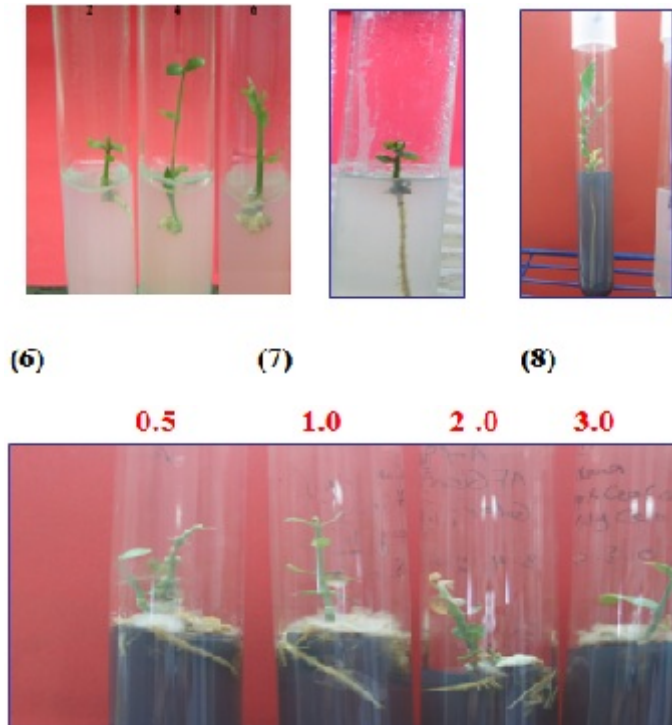
#### **The Role of Activated Charcoal (AC) in Jojoba:**

AC induced root initiation and elongation was observed in jojoba plantlet explants when cultured on MS medium fortified with 0.5, 1.0, 2.0 and 3.0 g/l AC Fig (8 and 9). Root induction from plantlet explants of jojoba had been existed by using MS medium supplemented with 5mg/l IBA and the shoots were subcultured on modified MS medium supplemented with 0.5, 1.0, 2.0 and 3.0 g/l AC to simultaneously promote both elongation and rooting and the best media was MS medium supplemented with 1.0 g/l AC & 2.0 g/l AC

combined with 5mg/l IBA (fig.9), similar to the results obtained with *Exacum* sp. (Unda *et al.*, 2007) . In *Eucalyptus* sp. the shoots regenerated from calli were subcultured to shoot elongation medium consists of MS medium containing 1 g/l AC (Barrueto Cid *et al.*, 1999). 100% rooting was induced by using this method depending on genotype of jojoba.

Successful rooting of camphor tree (*Cinnamomum camphora*) shoots derived from shoot tips and nodal segments were reported on WPM medium supplemented with AC, IBA and NAA. 100% of the shoots rooted on IBA and 2 g/l AC containing medium (Nirmal Babu *et al.*, 2003).

For *in vitro* rooting, the *H. abyssinica* shoots were kept in the dark for 4 days followed by transfer of shoots to WPM basal medium with 0.03 g/l AC. 100% rooting was induced by using this method depending on genotype (Feyissa *et al.*, 2005).



**Fig. 9:** (6, 7, 8 and 9): (6) Rooting was induced directly at the base of the shoot on 1,2,4,5 mg /l IBA. (7) MS+ 5.0 mg /l IBA, which proved to be the best rooting medium and 8) An *in vitro*-developed root 6 weeks after transfer to MS+IBA (0.5 mg l<sup>-1</sup>) but the root was unhealthy. (9) The shoot has developed healthy roots directly from the cut, basal end with 0.5, 1.0,2 .0 and 3.0 g/l AC.

#### **Transplantation:**

Following the protocol described under Materials and Methods, 40 plants from MS+IBA (5.0 mg l<sup>-1</sup>) were transferred out of culture. Of these, 35 plants survived (Fig. 11.1). Thus, transplantation survival of micropropagated plants was 75%.

The present study, thus, demonstrated the possibility for mass clonal propagation of a jojoba male and female by nodal segment cultures. MS medium supplemented with BAP (1 mg l<sup>-1</sup>) and CH (500 mg l<sup>-1</sup>) medium was used for recurrent shoot multiplication at a rate of 7–18-fold every 5 week and this rate of shoot multiplication was maintained for almost 2 years. The shoots could be readily rooted with a frequency as high as 75%. Transplantation survival of these plants was more than 75% (Fig. 11.2 and 11.3).

#### **Acclimatisation:**

*In vitro* plantlets with obvious roots were randomly selected and transferred to different sterile potting media, as showed in (Fig. 12.1 and 12.2). Transplanted plantlets were exposed to a regime gradually decreasing humidity for a period of 20 days, with light at 60-80 J.E m<sup>-2</sup> s<sup>-1</sup> from Phillips fluorescent day light tubes. The regime of humidity was managed with the help of nylon bags (Fig 11.2) which initially covered all the plantlet and was gradually removed for increasing duration of exposure to the air. Other cultural conditions

were as described above. After 70 days, the plants were transferred to soil. The number of nodes per explant (growth rate) and plant survival were evaluated at 15, 30, 45, 60, and 70 days. The potting media used were peat (turf), soil (A horizon from a typical Argiudol), sand, perlite, and a commercial organic mixture Agrosoil.

**(11.1)**



**(11.2)**



**(11.3)**

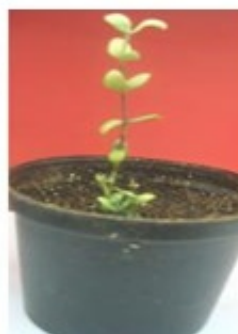


**Fig. 11:** (1,2 and 3) Hardened micropropagated plants 8 months after transfer to soil.

**(12.1)**



**(12.2)**



**Fig. 12:** (1 and 2) Hardened plants after transfer to greenhouse.

Acclimatisation of micropropagated jojoba plantlets for transfer to field has been difficult (Lee 1988; Zunino 1988; Chaturvedi & Sharma 1989), since the necessary high humidity environment can result in lack of aeration and death of the root system (Chaturvedi & Sharma 1989), or because the leaves produced in culture are reduced size (Lee 1988).

The Agrosoil™ potting medium gave good results for acclimatisation, perhaps because its porosity permitted better aeration. The organic matter present in this potting mixture might also promote rapid shoot growth.

The above cited authors reported the response to different growth regulators in micropropagation of jojoba but they did not study the genotype effect.

The described medium is suitable as a starting point for all the genotypes tested but each clone needs research to optimise its *in vitro* propagation.

#### ACKNOWLEDGMENTS

This work was supported by NRC. The authors are grateful to Dr. Nabil Elmogy for providing the semi-hardwood and seeds of jojoba plants. The authors are grateful also to Dr. Ahmed Magdi for his effort during the lab work.

**Abbreviations:**

BAP, 6-benzylamino purine; CH, casein hydrolysate; GA<sub>3</sub>, gibberellic acid; IAA, indole-3-acetic acid; IBA, indole-3-butyric acid

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