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# Genotype- and sex-specific protocols for *in vitro* micropropagation and medium-term conservation of jojoba

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## Abstract

Nodal explant cultures from field-grown five jojoba genotypes (EC 99690, EC 99692, EC 99692, EC 267779 and EC 171284; male and female plants), could be established on Murashige and Skoog (MS) medium. The nodal explants of different genotypes as well as sex elicited differential requirements of $N^6$-benzyladenine (BA) for optimum shoot regeneration and medium-term conservation. Female nodal explants of EC 99692 produced maximum shoots (10 shoots per explant) followed by male of EC 171284 (9.3 shoots per explant) on MS + 10 μM BA. The pulse treatment of 50 μM indole-3-butyric acid for 20 min caused *in vitro* rhizogenesis in 44 - 67 % cultures of various genotypes tested. A significant difference was observed for the conservation period of male and female cultures of all the genotypes. MS + 10 μM BA supported the shoot cultures of EC 99690, EC 99691 and EC 267779 for maximum conservation period, while MS + 5 μM BA proved to be optimum for conserving the shoots of EC 99692 and EC 171284. Generally, the female shoot cultures of genotypes survived for longer period than male ones.

*Additional key words*: clonal propagation, female, germplasm, male, *Simmondsia chinensis*, tissue culture.

## Introduction

*Simmondsia chinensis* (Link) Schneider, popularly known as 'jojoba' or 'hohoba', belongs to family *Simmondsiaceae*. It is an evergreen, dioecious desert shrub, native of Sonoran desert, North-west Mexico and Baja California (Benzioni 1995). The seeds storing liquid wax are used in cosmetic, pharmaceutical and plastic industries (Benzioni 1995). The vegetative propagation of jojoba *via* conventional stem cuttings did not prove effective due to long procedure and slow growth (Gentry 1958, Lee *et al*. 1985, Benzioni 1995). Due to its dioecious nature, it is important to plant sexually-known clones, therefore, micropropagation using tissue culture techniques could be the other alternative for production of desired sex-specific clones for its commercial cultivation. Several reports on *in vitro* micropropagation appeared in literature using various explants such as axillary buds (Chaturvedi and Sharma 1989, Llorente and Apostolo 1998, Khanam *et al*. 1999, Agrawal *et al*. 2002), shoot tips (Sardana and Batra 1998) and leaf tissues (Hamama *et al*. 2001). These reports are based on 1 or 2 genotypes and mostly do not document the effects of genotype and sex on micropropagation and germplasm conservation. In the present paper, we report *in vitro* micropropagation and conservation protocols applicable to 10 genotypes of *S. chinensis*.

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*Abbreviations*: BA - $N^6$-benzyladenine; IAA - indole-3-acetic acid; IBA - indole-3-butyric acid; MS medium - Murashige and Skoog (1962) medium; NAA - α-naphthalene acetic acid.
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## Materials and methods

**Establishment of cultures for shoot regeneration:** Healthy twigs (20 - 30 cm long) of 10 genotypes (5 each of females and males) of *Simmondsia chinensis* (Link) Schenider (EC 99690, EC 99691, EC 99692, EC 171284 and EC 267779) grown at Experimental Farm of National Bureau of Plant Genetic Resources, Jodhpur, were excised from 18- to 20-year-old plants. The twigs were thoroughly washed under running tap water for 30 min. After defoliating, nodal segments (1 - 2 cm) were cut and surface sterilized with 0.2 % (m/v) $HgCl_2$ for 10 min and finally rinsed 4 or 5 times with sterile distilled water (Tyagi and Prakash 2001). The cut ends of the explants those turned brown were trimmed prior to culture for their establishment on Murashige and Skoog (MS) medium (1962). Thereafter, the nodal explants were excised from 60-d-old *in vitro*-raised shoots on above medium and further cultured on MS medium supplemented with various concentrations (0, 2, 5, 10 μM) of $N^6$-benzyladenine (BA) to study shoot regeneration and conservation. MS basal medium served as the control. All the media were solidified with 0.8 % agar (*Qualigens, Glaxo Fine Chemicals*, Mumbai, India). The pH of the media was adjusted to 5.8 with 1 M HCl or 1 M NaOH prior to autoclaving. The culture vessels (25 × 150 mm; *Borosil*, Mumbai, India) containing 20 $cm^3$ media enclosed with polypropylene caps were autoclaved at 121 °C and 1.06 kg $cm^{-2}$ for 15 min. All the cultures were incubated in a culture room at temperature of 25 ± 2 °C under continuous light (40 μmol $m^{-2}$ $s^{-1}$) provided by cool white fluorescent tubes (40 W, *Philips*, Mumbai, India).

**Conservation:** Shoots regenerated on MS + 0 - 10 μM BA were allowed to grow on their respective parent culture media without transfer or subculture and studied for conservation. The period of conservation was computed from initiation of nodal explant cultures for shoot regeneration to survival of 50 % shoots in a culture (without any subculture).

**Rooting:** For root induction, 1 - 2 cm long female and male shoots of EC 99692 were excised and cultured on full- and half-strength MS medium supplemented with various concentrations (0, 1, 5, 10, 20, 30 μM) of indole-3-acetic acid (IAA), indole-3-butyric acid (IBA) and α-naphthalene acetic acid (NAA). On the basis of the observations of pilot experiments, the *in vitro*-regenerated shoots were given a pulse treatment of 50 μM IBA for 1, 10 and 20 min and vertically implanted on semi-solid MS + 10 μM IBA + 0.5 % activated charcoal medium for induction of roots. After 30 d, shoots with small roots, more suitable plantlets, were transferred to MS liquid medium (devoid of iron) supplemented with 5 μM BA and containing cotton as support matrix in the culture vessels (25 × 150 mm; *Borosil*) to facilitate the elongation of roots as well as shoots.

**Hardening and acclimatization:** A total of 104 plantlets (52 each of female and male) of EC 171284 were transferred to glass bottles containing *Soilrite* - *Pelrite* + *Irish peat moss* + *Vermiculite* (1:1:1) (*Kelpelrite*, Bangalore, India). The plants were kept under light provided by incandescent bulb with the irradiance of 40 μmol $m^{-2}$ $s^{-1}$ at 25 ± 2 °C under 16-h photoperiod. The bottles were closed during the first week of transfer of plants to maintain high air humidity (80 - 90 %). The caps of bottles were slightly removed after 7 d and completely removed after 15 d to expose the plants to *ex vitro* conditions. After 21 d the plantlets were transplanted to soil.

**Statistical analysis:** Number of shoots per culture and percentage of rooting cultures were recorded at intervals of 30 d. All the experiments were replicated three times and data are mean values of 30 explants for each experiment. Analysis of variance (*ANOVA*) was computed using a standard software applicable to randomized complete block design for genotype, sex and treatments.

## Results and discussion

**Establishment of cultures:** Some 25 - 52 % healthy nodal segment cultures of 10 genotypes were established on MS medium; a maximum of 52 % cultures of females of EC 99692. Generally, single shoot developed from axillary bud of nodal explants and grew further. After 60 d, the shoot had 3 - 5 nodes, which were excised and cultured on defined media for further experiments. Low percentage of culture establishment was reported by Chaturvedi and Sharma (1989) and 40 % by Mills *et al.* (1997).

Irrespective of sex and genotype, direct (without callus) single shoot bud developed from each axil of nodal explants within 10 - 12 d from culture on MS medium. No further increment was observed in 90-d-old cultures on MS basal medium in terms of number of shoots per culture. All the tested concentrations of BA enhanced the shoot proliferation. Considering all genotypes together, number of shoots per culture ranged from 1.7 to 10. For three genotypes of female (EC99691, EC99692 and EC171284) and two of male (EC99692 and EC171284), 10 μM BA proved optimum for shoot regeneration (Table 1). In 90-d-old cultures, female cultures of EC 99692 produced the maximum of 10 shoots per culture on MS + 10 μM BA. Shoot length

Table 1. Effect of different concentrations (0 - 10 μM) of BA on shoot regeneration from nodal segments of jojoba after 90 d in culture. Mean ± SE, $n = 30$.

| Genotype | Number of shoots [explant$^{-1}$] | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | female | | | | male | | | |
| | 0 | 2 | 5 | 10 | 0 | 2 | 5 | 10 |
| EC 99690 | 2.6 ± 0.12 | 2.7 ± 0.09 | 4.6 ± 0.03 | 3.4 ± 0.13 | 2.3 ± 0.10 | 1.8 ± 0.03 | 5.6 ± 0.06 | 5.0 ± 0.06 |
| EC 99691 | 2.4 ± 0.08 | 3.4 ± 0.13 | 5.0 ± 0.06 | 8.7 ± 0.10 | 1.7 ± 0.09 | 4.6 ± 0.22 | 5.5 ± 0.17 | 3.0 ± 0.10 |
| EC 99692 | 3.0 ± 0.33 | 4.7 ± 0.14 | 5.3 ± 0.13 | 10.0 ± 0.20 | 2.2 ± 0.11 | 4.7 ± 0.20 | 3.7 ± 0.04 | 7.4 ± 0.20 |
| EC 171284 | 2.5 ± 0.08 | 2.0 ± 0.07 | 3.7 ± 0.21 | 7.0 ± 0.16 | 1.6 ± 0.22 | 6.1 ± 0.32 | 6.0 ± 0.19 | 9.3 ± 0.25 |
| EC 267779 | 2.0 ± 0.07 | 5.2 ± 0.14 | 8.2 ± 0.21 | 6.3 ± 0.26 | 2.5 ± 0.13 | 6.3 ± 0.22 | 4.2 ± 0.21 | 4.6 ± 0.13 |

ranged from 0.9 to 2.5 cm in female cultures and 1.0 to 2.7 cm in male cultures. Maximum 2.5 cm shoots of female and 2.7 cm of male cultures of EC 267779 were recorded on MS + 10 μM BA and MS + 2 μM BA, respectively. The effectiveness of BA for micropropagation of jojoba has already been documented (Llorente and Apostolo 1998, Roussos *et al.* 1999). Llorente and Apostolo (1998) reported 4.6 shoots per culture in 30-d-old cultures on MS + 1 mg dm$^{-3}$ BA. Micropropagation from nodes and shoot tips of seedling plants has been reported by Roussos *et al*. (1999) wherein 8.9 shoots per culture on Driver and Kyuniki (1984) walnut medium supplemented with 19.7 μM BA were obtained in 8-week-old cultures. However, Chaturvedi and Sharma (1989) and Mills *et al*. (1997) concluded that auxin-cytokinin combinations (*i.e.* BA + IAA and 2iP + IAA, respectively) are better than individual cytokinin for shoot regeneration in jojoba.

In 90-d-old cultures, mean number of shoots per culture ranged from 2 to 10 in females and from 1.6 to 9.3 in males on BA-supplemented media (Table 1). Considering the overall response of shoot regeneration in female and male genotypes, three female genotypes of EC 99691, EC 99692 and EC 267779 responded better than male ones; converse is true for EC 99690 and EC 171284. Gui (1979) in *Actinidia chinensis*, De Winnaar (1988) in *Carica papaya* and Mehra and Cheema (1985) in *Populus deltoides* reported the better shoot regeneration in females than males. On contrary, Litz and Conover (1981) reported explants from male plants are more responsive compared to female ones. This phenomenon could be attributed to the endogenous content of cytokinins (Dauphin-Guerin *et al*. 1980) and inherent variations among the genotypes of different sex. However, no such difference was observed in morphogenic potential in the explants derived from two sexes of *Phoenix dactylifera* (Tisserat *et al*. 1979). *ANOVA* analysis shows the significant differences at ≤ 1 % level for the number of shoots per culture for genotypes, treatments and interactions (Table 2).

Table 2. Analyses of variance (*F*-values) of the effects of genotype, sex, treatments and their interactions on number of shoots per culture and percentage of shoot regenerated roots (*,** - significantly different at $P \leq 0.05$ and $P \leq 0.01$, respectively).

| | Number of shoots [explant$^{-1}$] | Shoots regenerated roots [%] |
|---|---|---|
| Genotype | 489.8** | 3.7** |
| Sex | 0.22 | 58.0** |
| Treatment | 1430.3** | 1020.2** |
| Genotype × sex | 259.5** | 10.6** |
| Genotype × treatment | 130.1** | 1.7 |
| Sex × treatment | 122.7** | 9.0** |
| Genotype × sex × treat. | 59.1** | 1.9* |

Table 3. Effects of full-strength and half-strength MS medium and auxins in concentrations 0 - 30 μM on root induction in shoots of jojoba (EC 99692). No roots were observed on basal MS medium.

| | | Female | | | | | Male | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 1 | 5 | 10 | 20 | 30 | 1 | 5 | 10 | 20 | 30 |
| 1 MS | IAA | 0 | 0 | 25 | 15 | 25 | 4 | 10 | 20 | 15 | 18 |
| | IBA | 10 | 24 | 30 | 12 | 20 | 10 | 10 | 24 | 30 | 20 |
| | NAA | 10 | 18 | 30 | 50 | 15 | 8 | 4 | 18 | 25 | 20 |
| 1/2 MS | IAA | 8 | 12 | 20 | 16 | 30 | 0 | 15 | 12 | 20 | 15 |
| | IBA | 8 | 18 | 30 | 24 | 30 | 8 | 19 | 20 | 33 | 24 |
| | NAA | 4 | 18 | 10 | 20 | 15 | 12 | 8 | 25 | 33 | 36 |

**Rooting:** Rooting occurred on IAA- and NAA-supplemented media with profuse callus. But IBA (10 μM) was found optimum to induce 30 % rooting in female shoots and 24 % in male shoots with a little or no callus. No rooting was observed on full- and half-strength MS media devoid of auxins. Callus formation at the base of shoots is not desirable for successful transplantation of the plantlets as it obstructs the connection between shoots and roots and finally survival to hardening of the plants (Thorpe *et al*. 1991). On the basis of the preliminary observations two higher concentrations of IBA *i.e.* 25 and 50 μM were tested further as pulse treatments of various duration (0 - 20 min). Irrespective of genotype, sex and duration of pulse treatment, shoots of all genotypes developed roots directly (without callus) within 30 d on MS + 10 μM IBA + 0.5 % activated charcoal (Table 4). Root regenerating cultures ranged from 44 to 67 % in females and 44 to 54 % in males. Shoots of female genotype of EC 99691 regenerated roots in 67 % cultures on MS + 10 μM IBA + 0.5 % activated charcoal after 20 min pulse treatment of 50 μM IBA (Table 4). Rooting in jojoba has been an impediment for successful *in vitro* micropropagation protocols (Chaturvedi and Sharma 1989, Apostolo *et al*. 2001). As low as 25 % shoots of jojoba regenerated roots *in vitro* on MS + IBA (Llorente and Apostolo 1997). Several authors have reported the improvement in rooting in shoots of jojoba *in vitro*. Mills *et al*. (1997) reported that addition of IAA and IBA increased the rooting to 50 - 60 % of shoot cultures. Chaturvedi and Sharma (1989) observed as high as 90 % rooting of *in vitro*-raised shoots on Schenk and Hildebrandt (1972) medium containing IBA + NAA + caffeic acid. Rost and Hinchee (1980) reported a two-step (pulse of IBA + polyvinylpyrrolidone) method for root development. Further, pulse treatment of cyclodextrins (0.03 - 0.5 mM) along with 0.015 mM IBA could increase the rooting up to 80 % in jojoba (Apostolo *et al*. 2001). In our studies, on transfer of plantlets to MS liquid medium (devoid of iron) containing 5 μM BA and cotton as support matrix, shoots as well as roots elongated from 2 to 4.5 cm and from 1.7 to 3 cm, respectively, in 60-d-old plantlets. *ANOVA* shows the significant difference for genotype, sex, treatment and their all interactions (Table 2).

Table 4. Effects of pulse treatment of IBA (50 μM) duration (1 - 20 min) on shoots regenerated roots [%] of jojoba cultured on MS + 10 μM IBA + 0.5 % activated charcoal after 30 d from transfer onto rooting medium.

| | Female | | | Male | | |
|---|---|---|---|---|---|---|
| | 1 | 10 | 20 | 1 | 10 | 20 |
| EC 99690 | 21 | 40 | 50 | 25 | 37 | 54 |
| EC 99691 | 25 | 42 | 67 | 25 | 27 | 53 |
| EC 99692 | 35 | 40 | 50 | 30 | 28 | 54 |
| EC 171284 | 30 | 38 | 44 | 25 | 33 | 44 |
| EC 267779 | 25 | 37 | 45 | 18 | 30 | 50 |

**Conservation:** In female and male shoot cultures, the development of new shoots was accompanied by the senescence of existing shoots on all the media tested under *in vitro* conservation. On BA-supplemented media, the cultures remained viable for a longer duration compared to that on basal MS medium. The conservation period varied from 270 - 480 d depending upon the sex and genotype (Fig. 1). Of all the genotypes studied, female and male cultures of EC 171284 could be stored for a maximum period of 480 and 420 d, respectively, on MS + 5 μM BA (Fig. 1). In general, the female shoot cultures of all genotypes were conserved for longer duration than male ones. Amongst the various concentrations of BA tested, 10 μM BA was found optimum for conservation of EC99690, EC99691 and EC267779 and 5 μM BA for EC 99692 and EC 171284. Of 104 plants (52 each of female and male) of EC171284 were transplanted to soil, 55 % plants of female and 37 % of male survived in nature, indicating the successful recovery of functional plants after medium-term conservation of jojoba genotypes using tissue culture methods.

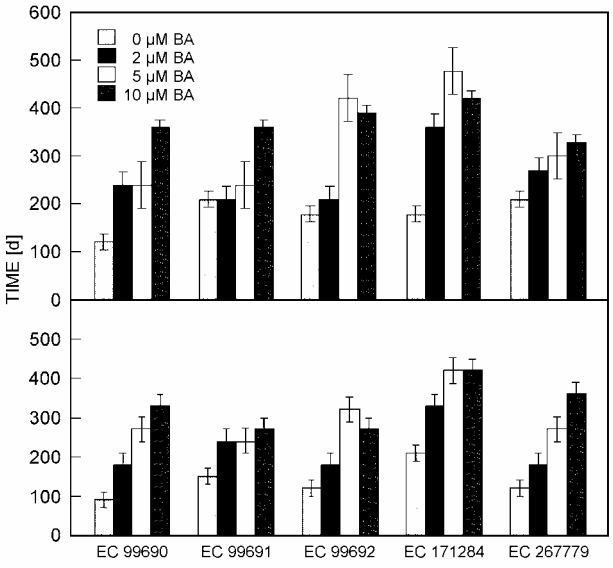


Fig. 1. Conservation period of shoot cultures (survival of 50 % shoots) of different female (*above*) and male (*below*) genotypes of jojoba on MS + BA. Means ± SE, $n = 30$.

The present study describes the protocols for micropropagation and medium-term conservation of 10 female and male genotypes of jojoba. Following these protocols, all the genotypes are being conserved for about last two years in our laboratory by subculturing/ transfering onto fresh defined media. The maintenance of tissue cultures for a prolonged period is difficult as the number of cultures of new germplasm accessions keep on

adding and become unmanageable. Therefore, the work is under progress to standardize the cryopreservation protocols using encapsulation-dehydration and vitrification techniques for long-term conservation.

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