

In Vitro Propagation of Jojoba

Berta E. Llorente and Nancy M. Apóstolo

Abstract

Jojoba (*Simmondsia chinensis* (Link) Schn.) is a nontraditional crop in arid and semi-arid areas. Vegetative propagation can be achieved by layering, grafting, or rooting semi-hardwood cuttings, but the highest number of possible propagules is limited by the size of the plants and time of the year. Micropropagation is highly recommended strategy for obtaining jojoba elite clones. For culture initiation, single-node explants are cultivated on Murashige and Skoog medium (MS) supplemented with Gamborg's vitamins (B5), 11.1 μM BA (N_6 -benzyl-adenine), 0.5 μM IBA (indole-3-butyric acid), and 1.4 μM GA_3 (gibberellic acid). Internodal and apical cuttings proliferate on MS medium containing B5 vitamins and 4.4 μM BA. Rooting is achieved on MS medium (half strength mineral salt) amended with B5 vitamins and 14.7 μM IBA during 7 days and transferred to develop in auxin-free rooting medium. Plantlets are acclimatized using a graduated humidity regime on soil: peat: perlite (5:1:1) substrate. This micropropagation protocol produces large numbers of uniform plants from selected genotypes of jojoba.

Key words: Micropropagation, Plant growth regulators, Plant tissue culture, *Simmondsia chinensis*

1. Introduction

Simmondsia chinensis (Link) Schn. (jojoba) is a nontraditional crop in arid and semi-arid areas. It is naturally well adapted to saline soils and high temperature environmental conditions (1). Jojoba seeds store lipids in the form of liquid wax that makes up 40–60% of their dry weight. This wax and its derivatives have potential for wider applications in cosmetics, pharmaceuticals, lubricants, extenders, and antifoaming agents (2, 3). There is an increased interest in the agricultural production of jojoba and more promising experience has accumulated every year respecting cultivation requirements, planting densities, management practices, productivity, propagation techniques, and genetic improvement (1, 2).

Jojoba is dioecious and unable to initiate sexual reproduction before flowering (usually 2–4 years from germination) (1).

Propagation by direct seeding has genetic heterogeneity and half of the seedlings are males. However, 8–10% males are necessary for pollination (4). Setting up a plantation with asexual propagules is more expensive than with seed, and saves time in replanting plants as well as crop produced of known sex and lineage. Vegetative propagation can be achieved by layering, grafting, or rooting semi-hardwood cuttings, but the highest number of propagules is limited by plant size and time of year (2, 4).

Micropropagation of elite individuals exploits totipotency of plant cells and offers a promising means of commercial mass production of pathogen-free superior clones. In vitro-derived jojoba plants grow more vigorously than both seedlings and rooted cuttings, and are significantly larger after the first year of growth (1, 2, 4, 5).

Some protocols for in vitro culture of jojoba are known. There are reports of jojoba somatic embryogenesis from zygotic embryo (6, 7) and leaf explants (7, 8). A number of researchers have described in vitro culture of single-node explants using both axillary and apical buds (2, 5, 9–15).

Shoots exhibited differential morphogenic behavior under the influence of growth regulators and adjuvants during in vitro propagation (9–11). Variations in the response of the explants have been observed in terms of percentage of shoot regeneration, proliferation rate, shoot length, callus presence, and rooting behavior (9–11). Some genotypes exhibited 75% root formation while others displayed scarce rooting in medium with 6 μM indole-3-butyric acid (IBA) (9) and the pulse treatment of 50 μM IBA caused 44–67% in vitro rhizogenesis of various genotypes tested (11). On the other hand, no significant difference in bud initiation, rooting and survival in greenhouse was observed in some genotypes studied (14).

A basic protocol for jojoba micropropagation suitable as a starting point for all genotypes is described in this chapter. However, it is recommended to develop an optimized protocol for each genotype because in vitro response of each clone can be unpredictable.

2. Materials

1. Systemic and contact fungicidal solution (4% Metalaxil-M + 64% Mancozeb, Fich M™, Cheminova Agro de Argentina S.A.) diluted to 0.2% with tap water.
2. Sodium hypochlorite solution (commercial bleach solution 5.5 g/L active chlorine).
3. Surfactant Tween 20 solution 0.1% (v/v).

4. Autoclaved distilled water, 150 mL aliquots in 250 mL screw-capped bottles.
5. Doubled distilled water.
6. Basal medium (BM) contained Murashige and Skoog salts (MS) (16) and B5 Gamborg's vitamins (17) (Table 1).
7. d-sucrose pure.
8. Agar (e.g., plant cell culture tested from Sigma-Aldrich).
9. N₆-benzyl-adenine (BA).
10. Indole-3-butyric acid (IBA).

Table 1
Plant culture medium

MS salts (16)	mg/L^a	mg/L^b
KNO ₃	1,900	950
NH ₄ NO ₃	1,650	825
MgSO ₄ ·7H ₂ O	370	185
KH ₂ PO ₄	170	85
CaCl ₂ ·2H ₂ O	440	220
H ₂ BO ₃	6.2	3.1
MnSO ₄ ·H ₂ O	16.9	8.5
ZnSO ₄ ·7H ₂ O	8.6	4.3
Na ₂ MoO ₄ ·2H ₂ O	0.25	0.125
CuSO ₄ ·5H ₂ O	0.025	0.012
CoCl ₂ ·6H ₂ O	0.025	0.012
KI	0.83	0.415
FeSO ₄ ·7H ₂ O	27.8	13.9
Na ₂ EDTA·2H ₂ O	37.3	18.6
Sucrose	30,000	20,000
Gamborg's B5 vitamins (17)		
Thiamine·HCl	10	10
Pyridoxine·HCl	1	1
Nicotinic acid	1	1
Myo-inositol	100	100
pH	5.8	5.8

^aInitiation and proliferation media

^bRooting medium

11. Gibberellic acid (GA_3)
12. KOH solutions 0.1, 0.5, and 1.0 M.
13. HCl solutions 0.1, 0.5, and 1.0 M.
14. Flat-bottom glass tubes 55 mL capacity (2.7×10 cm).
15. Potting media, consisting of soil (a horizon from a typical Argiudol), peat (commercial turf Terrafertil™) and perlite in a 5:1:1 by volume.
16. Glass flasks 350 mL (6.4×11 cm).
17. Screw cap tubes.
18. Sterile Petri dishes (9 cm in diameter).
19. Beaker $\times 1,000$ mL.
20. Scalpels, forceps, spirit burner to flame sterilize instruments.
21. Pruning shears.
22. Laboratory transparent film (30–150 μ m in thickness).
23. Transparent plastic bags.
24. Plastic pots (8 and 12 cm in diameter).
25. Magnetic stirrer, magnetic bar.
26. Microwave cooker.
27. Laminar flow bench.
28. Culture room or growth chamber.
29. Glasshouse.

3. Methods

This protocol has six main stages: (1) preparation and sterilization of culture media; (2) surface sterilization of plant source material; (3) establishment of axenic cultures of stem nodal explants (culture initiation); (4) shoot proliferation; (5) rooting of in vitro shoots; and (6) acclimatization of regenerated shoots in ex vitro conditions.

3.1. Preparation and Sterilization of Stock Solutions and Culture Media

1. Prepare BM medium in doubled distilled water supplemented with 3% (w/v) sucrose. Store at 4°C.
2. Prepare IBA and GA_3 stock solutions by dissolving the powder in a few drops of KOH 0.1 M and add doubled distilled water to make up the volume to 1.0 mM. Store at –20°C.
3. Prepare BA stock solution: Dissolve powder in a few drops of HCl 0.1 M and add doubled distilled water to make up the volume to 1.0 mM. Store at –20°C.

4. Initiation medium: Use BM, supplemented with 11.1 μM BA, 0.5 μM IBA, and 1.4 μM GA₃ from stock solutions.
5. Proliferation medium: To BM add 4.4 μM BA from stock solution.
6. Rooting medium (RM): Reduce concentration of MS mineral salts to half and sucrose to 2% (w/v).
7. Root induction: Transfer shoots to RM containing 14.7 μM IBA and for root development use hormone-free RM medium.
8. Adjust the pH of the media to 5.8 with HCl or KOH (1.0, 0.5, or 0.1 M).
9. Add agar 0.7% (w/v) for the initiation and proliferation media and 0.6% (w/v) for rooting media. Dissolve by microwaving.
10. Dispense the media into suitable containers, 15 mL aliquots into 55 mL capacity flat-bottom glass tubes for the shoot initiation and rooting medium, or 60 mL aliquots into 300 mL glass flasks for shoot proliferation.
11. Sterilize the media by autoclaving at 121°C for 20 min (101 kPa steam pressure).
12. Store the autoclaved media at room temperature in the dark for a maximum of 20 days.

3.2. Surface

Sterilization of Plant Source Material

1. Choose healthy plants of known sex.
2. Spray the parental stock plants with fungicidal solution 48 h before initiating tissue culture (see Note 1).
3. Cut stem nodal sections (3–6 nodes from apex) of actively growing adult jojoba plants (see Note 2).
4. Cut single-node segments with two axillary buds or one apical bud from stem nodal section.
5. Wash nodal sections for 2 h under running tap water, predisinfect with 2 g/L fungicide (Fich M™); and agitate in a sterile beaker on a magnetic stirrer for 60 min (see Note 3).
6. Disinfect stem segments by immersion in 20% commercial sodium hypochlorite solution, added with two drops of surfactant, and agitate with a magnetic stirrer for 30 min.
7. Sterilize the laminar flow surface by 70% ethanol before use.
8. Rinse in sterile distilled water three times to remove the sterilizing agent under laminar flow.
9. Store in sterile distilled water until culture initiation (no longer than 1 h), under laminar flow.

3.3. Environmental Conditions for Culture

1. Incubate in vitro cultures in a growth room at 24 ± 2°C under Phillips fluorescent daylight tubes (30–40 $\mu\text{mol}/\text{m}^2\text{s}$) with a 16 h photoperiod.

2. At the acclimatization stage, incubate the regenerated *in vitro* plants in a growth room at $24 \pm 2^\circ\text{C}$, with a 16 h photoperiod and $40\text{--}50 \mu\text{mol}/\text{m}^2/\text{s}$ light intensity.
3. Glasshouse conditions oscillate from 0 to $300 \mu\text{mol}/\text{m}^2/\text{s}$ light intensities, $20\text{--}40^\circ\text{C}$ temperature and 95–40% relative humidity (night to day, respectively).

3.4. Initiation of Culture

1. Sterilize and flame all instruments and laminar flow surface.
2. Cut ends and white portions of the each axenic explant.
3. Eliminate apical cross section of the leaf blades (see Note 4).
4. Firmly insert the base of node segment into 15 mL initiation medium in a flat-bottom tube (see Note 5). A minimum of 30 explants per genotype is recommended for initiation (see Fig. 1a).
5. Seal the tube with transparent film.
6. Incubate in the growth room (see Fig. 1b, d).
7. Suggested parameters to evaluate: frequency rate of sprouting shoot, number of regenerated shoots per explant, shoot length, presence of callus, and number of hyperhydric plantlets for each genotype.
8. When the shoots grow, transfer to the proliferation medium (4–6 weeks).

3.5. Shoot Proliferation

1. Sterilize and flame all instruments and laminar flow before use.
2. Select well developed shoots from initiation culture (see Fig. 1d).
3. Remove hyperhydric, malformed, oxidized and dead tissues (see Note 6). Excise callus and basal part of regenerated shoots (see Note 7).
4. Cut single-node segments with two axillary buds or one apical bud and transfer them to 350 mL flasks (8–10 shoots per flask) with 60 mL sterile proliferation medium (see Fig. 1e).
5. Seal the tube with plastic wrap (see Note 8).
6. Incubate shoots in a growth room and regularly subculture at 4–5 week interval to the same fresh medium for continued proliferation (see Fig. 1f).
7. Suggested parameters to evaluate: proliferation rate (relationship between final and initial node numbers), number of leaves, shoot length, percentage of hyperhydric shoots, and callus development.

3.6. Rooting

1. Sterilize and flame all instruments and laminar flow before use.



Fig. 1. Jojoba micropropagation. (a–d) Initiation culture; (e–g) Shoot proliferation; (h) In vitro rooting; (i, j) Acclimatization; (k) Micropropagated plants in glasshouse. Bars: a–e, g, h, j: 1 cm; f, i: 2 cm; k: 5 cm.

2. Select elongated shoots (4–5 cm) from proliferation culture (see Fig. 1g).
3. Remove hyperhydric, malformed, oxidized, and dead tissues. Excise basal callus (see Notes 6 and 7).
4. Insert the base of 3- to 5-node shoots into flat-bottom tube with 20 mL aliquots of rooting induction medium RM supplemented with 14.7 μM IBA.
5. Seal the tube with plastic wrap.
6. Incubate cultures during 7 days for root induction (“pulse treatment”).
7. Subculture shoots in a flat-bottom tube with 20 mL aliquots of RM root development medium devoid of plant growth regulators.
8. Seal the tube with plastic wrap.
9. After 6–8 weeks of culture, the regenerated shoots will develop root systems consisting of some long roots (see Fig. 1h).
10. Suggested parameters to evaluate: rooting percentage, root number per shoot and quality, root length, dry weights of shoots and roots, root surface area, time until the appearance of the first root (precocity) for each shoot and morphological features (see Notes 9 and 10).
11. Medium supplemented with oligosaccharides or plant growth-promoting rhizobacteria are alternative strategies to use with recalcitrant clones (see Notes 11–13).

3.7. Acclimatization to Ex Vitro Conditions

1. Remove each rooted shoot carefully from the culture medium and rinse off the agar by washing roots with tap water without injuring (see Fig. 1h).
2. Transfer plants individually into 8 cm diameter plastic pots, each containing the acclimatization mixture (see *Potting media in Materials*) (see Fig. 1i) and well irrigate them.
3. Cover all potted plants with transparent plastic bags and grow them under acclimatization conditions (see Fig. 1i).
4. After 7 days, cut off a corner from the top of each bag to reduce the relative humidity and facilitate slow acclimatization of the plants to ex vitro conditions.
5. Gradually remove the remaining top corner of the bags after a further 2 weeks and discard the bags after an additional week (see Fig. 1j). (see Note 14).
6. Transfer plants to 12 cm diameter plastic pots in the glasshouse.
7. After 4–6 weeks in the glasshouse, plants develop their characteristic ex vitro aspect (see Fig. 1k). Plants showed stems with

opposite leaves, buds, and branches. Secondary thickening was observed in main shoot. Expanded elliptic leaves have thick wax layer and gray-green in color. Roots were well developed.

8. Suggested parameters for weekly evaluation: survival, number of nodes and shoot length per plantlet, rates of proliferation and elongation.

4. Notes

1. The bacterial and mainly fungal contaminations are often major hurdle in establishing jojoba in vitro cultures and the losses reaching up to 90% (5). About 80% explants are successfully used for initiating in vitro cultures by using the disinfection protocol described here. Culture initiation from greenhouse-grown plant substantially reduces the contamination rates. Moreover, in vitro propagation by meristem culture is recommended for producing a large number of pathogen-free plants (18).
2. The explant collection time during the year is very crucial for the success of micropropagation. It is linked with the proliferation of microorganisms and the physiological condition of the plant which determines the degree of explant dormancy. In jojoba, the explants collected during spring show higher bud-break (15).
3. It is recommended that the protocol runs separately for each clone. Individual management of each clone permits the study of the in vitro responses of each genotype and reduces cross-contamination losses. Shoot proliferation, callus development, and hyperhydricity differ significantly among clones at all jojoba micropropagation stages (9–11, 13). Also, growth, flowering, wax production, and response to salinity are influenced by genotype (2, 3).
4. Cut leaf blades stimulate the sprouting of new shoots because the injury decreases leaf auxin synthesis, amending the local auxin/cytokinin ratio that stimulates the progression of buds from a transition stage to sustained bud outgrowth (19).
5. BA is more effective than kinetin (Kn) for inducing the growth of primary explants (9) (see Fig. 1a–d). Furthermore, nodal explants of different genotypes, as well as different sexes, may have differential requirements of cytokinins for shoot regeneration and medium-term conservation (11). In order to get the optimum jojoba proliferation in vitro, experiments with different proportions of sucrose and BA were conducted using the rotatable central composite design. This methodology uses

statistical and mathematical techniques for modeling and analyzing problems in which a response of interest is influenced by several variables. The best proliferation rate (5.7) is obtained in the presence of 2% sucrose and 7.2 μM BA (20).

6. Hyperhydricity is a severe problem in *in vitro* culture. The term has been adopted to describe shoots with a vitreous and deformed appearance (see Fig. 2b). Some of the causes that lead to hyperhydricity are environmental factors, gelling agent properties, and growth regulators (21). The anatomy of normal *in vitro* leaves from micropropagated jojoba displays stomata at epidermal level, guard cells with a cuticular border and a thin cuticle with epicuticular waxes in the leaf epidermis (see Fig. 2a, c, d, g). *In vitro* hyperhydric shoots and leaves present numerous anatomical defects, e.g., short internodes, mesophyll, and stem cortex hypertrophy, malformed nonfunctional stomata some of which are occluded by a cuticular plug, epidermal discontinuity, and xylem hypolignification (see Fig. 2b, e, f, h, i). Explants with these features cannot survive to acclimatization (22).
7. Adventitious shoot formation in callus tissue can produce somaclonal variation, which can be used to generate some useful genetic variation in commercial cultivars. However it must be prevented to occur with utmost care especially for clonal propagation of elite germplasm/genotypes (18).
8. Ventilation during the proliferation stage affects the development of jojoba shoots. Variation in the response depends on the genotype, the extent of ventilation, and the shoot subculture number. In the case of some genotypes, the micropropagation protocol should include Magenta® boxes equipped with vented lids (or equivalent gas-permeable containers) as growing vessels to improve growth and reduce hyperhydricity (23).
9. Pool jojoba clones cultured in medium supplemented with auxin during 45 days, showed the best rooting with 14.8 μM IBA (33% rooted plants). However, when is used only 7 days pulse IBA treatment (14.8 μM), 50% of plants rooted (9, 12).
10. Root surface area from *in vitro* plants can be determined by immersing air-dried roots in $\text{Ca}(\text{NO}_3)_2$ saturated solution and determine salt quantity (mg), removed from the solution, which is sticking to the root surface (24).
11. Trehalose, a nonreducing disaccharide, plays an important physiological role as a storage carbohydrate and protector from abiotic stresses. In the initiation stage, explants cultured in the hormone-free medium containing 1 mM trehalose show a significant increase in the proliferation rate (3.2) when compared with phytohormone control medium (2.4) (Fig. 3a). In the proliferation stage, a shoot proliferation

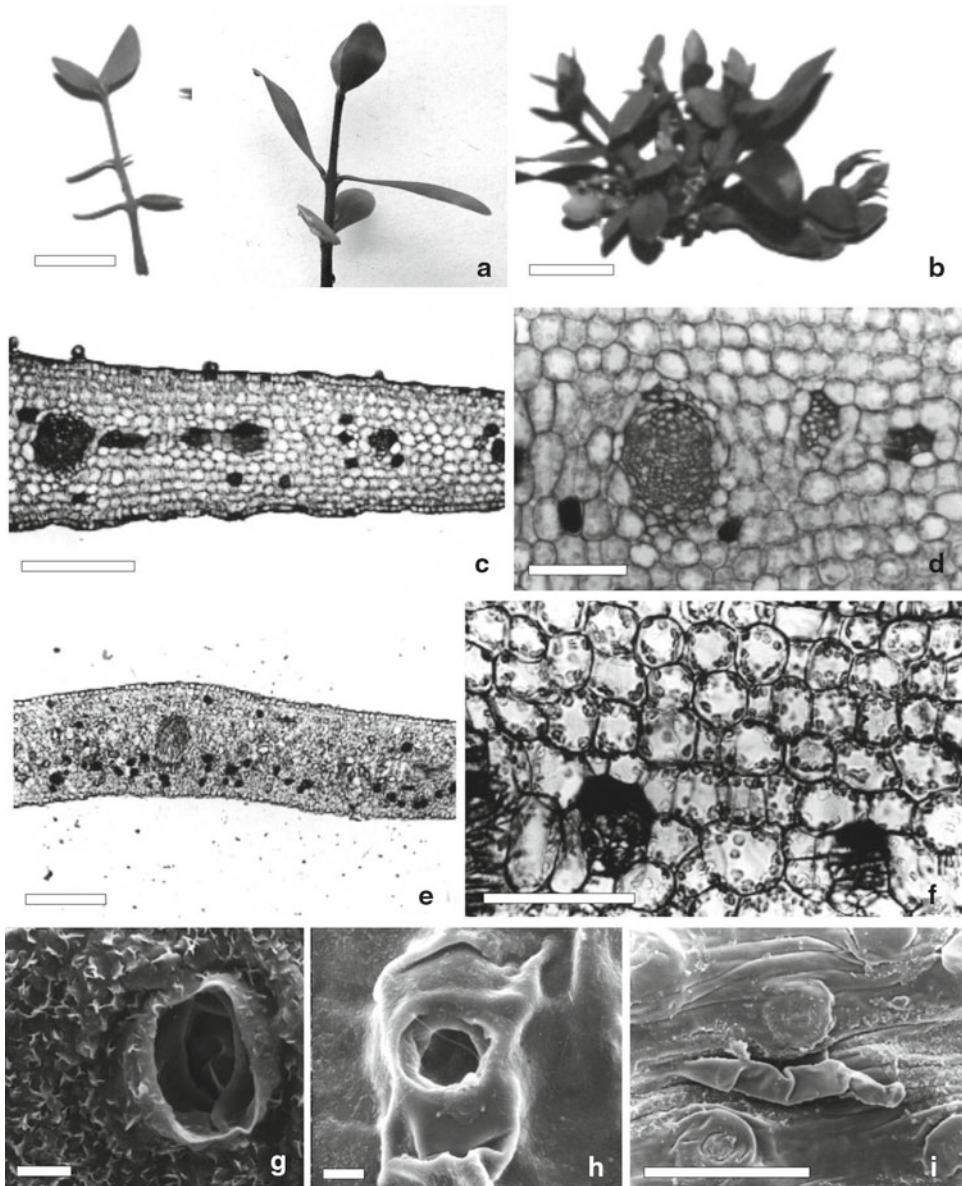


Fig. 2. Morphology and anatomy of in vitro jojoba in shoot proliferation. (a) Normal shoots; (b) Hyperhydic shoots; (c, d) Transverse section of normal leaves (LM); (e, f) Transverse section of hyperhydic leaves (LM); (g) Normal leaf surface, stomata (SEM); (h, i) Hyperhydic leaf surface (SEM). *LM* light microscopy; *SEM* scanning electron microscopy. Bars: a, b: 1 cm; c, e: 250 μ m; d, f: 50 μ m; g, h: 10 μ m; i: 100 μ m. (Figure 2g–i, reproduced from Llorente et al. (12) and Apóstolo and Llorente (22), with permission from Springer Science + Business Media BV).

rate of 4.8 is achieved in BM with 4.44 μ M BA and 1 mM trehalose. Also, the addition of trehalose to the IBA-rhizogenesis media decreases basal callus (12).

12. Addition of cyclodextrins to modified MS (16) culture medium containing IBA as 1 week pulse treatment doubles rooting

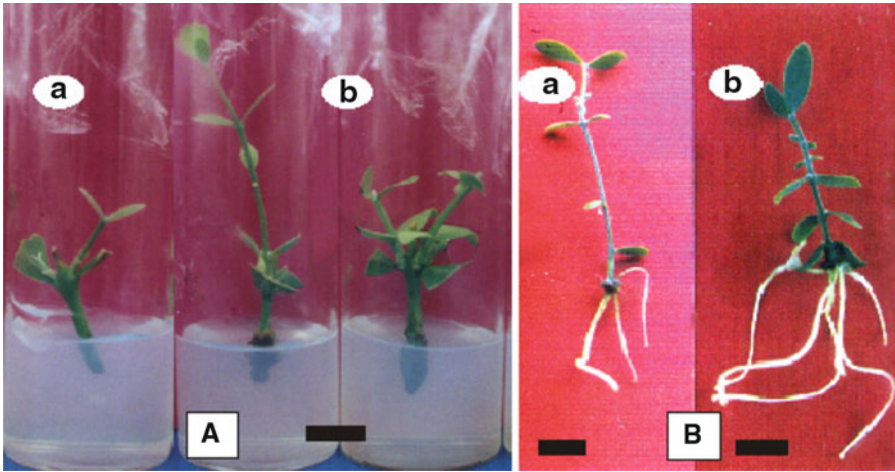


Fig. 3. Effect of oligosaccharides on jojoba micropropagation. (A) Trehalose effect on initiation cultures: (a) shoots in basal medium (BM) supplemented with $0.49 \mu\text{M}$ IBA, $1.44 \mu\text{M}$ GA_3 and $11.10 \mu\text{M}$ BA; (b) shoots in BM supplemented with trehalose 1 mM . (B) β -Cyclodextrins (β -Cd) effect on in vitro rooting: (a) shoot rooting in rooting medium (RM) supplemented with $15 \mu\text{M}$ IBA (control treatment); (b) shoot rooting in RM supplemented with $15 \mu\text{M}$ IBA and $30 \mu\text{M}$ β -Cd. Bars: a, b: 1 cm (Fig. 3 Ba, 3 Bb reproduced from ref. (25) with permission from Springer Science + Business Media BV).

percentage and induces earlier rooting with respect to IBA control medium (see Fig. 3b). Increase in rooting percentage after treatment with cyclodextrins was also observed in the absence of IBA, indicating that both compounds promote rooting per se (25).

13. A plant growth-promoting rhizobacterium (*Azospirillum brasilense* strain Cd) stimulates in vitro rooting of jojoba. Explants grown for 4 days on a medium supplemented with $14.8 \mu\text{M}$ IBA were transferred to hormone-free medium and inoculated at with 0.1 mL of bacterial culture at the base of each explant (2×10^7 colony forming units). The inoculated explant reached a rooting percentage of 86% and rooted 5 days earlier than the controls. Also, they showed a significant increase in the mean number of roots per shoot, and exhibited less callus production than the controls (19).
14. The transition from in vitro to ex vitro conditions can be difficult. Shoots growing in vitro have been continuously exposed to a microenvironment under low light intensity, aseptic conditions, and high humidity. The micropropagated plants undergo some anatomical and physiological changes. High humidity in the in vitro culture conditions should be gradually reduced to allow plantlets to develop normal cuticle and stomata and to avoid dehydration during acclimatization (26). Likewise, relatively low levels of light produce thin leaves and it is necessary to put them under the shade for 4 weeks and gradually move them to the ex vitro light level, especially during summer months (27).

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