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IMPROVEMENT OF *IN VITRO* PROPAGATION OF JOJOBA BY LOW CONCENTRATIONS OF NaCl OR MANNITOL

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This work was done to improve our understanding about the effect of growth regulators on micropropagation and gene expression of stress tolerant jojoba plant and how they were influenced by the presence of different stress agents. Genetic variation of plants obtained from seeds of jojoba, was checked using RAPD technique. The registered genetic variation was too high and to avoid it, offspring one shoot was used in this study. A combination of 3 mg/l BAP and 0.1 mg/l NAA was the best for jojoba micropropagation, while changing the concentrations from that decreased shoot multiplication and growth. Application of GA_3 is not recommended for *in vitro* multiplication of jojoba. In case of jojoba as stress tolerant plant species, the effect of the selected growth regulators combination (3 mg/l BAP + 0.1 mg/l NAA) on micropropagation was improved when high salt-MS medium containing low concentrations of NaCl or mannitol was used. The negative effect of NaCl or mannitol on *in vitro* shoot growth was only detected at concentration 4 g/l. On the other side, lead acetate abolished the effect of growth regulators on jojoba micropropagation even in low concentration. Isoenzymes and SDS-PAGEs indicated that the registered effects of growth regulators and stress agents on shoot multiplication and growth were associated with variation in gene expression.

Keywords: *In vitro* regeneration; Gene expression; RAPD; abiotic stress; *Simmondsia chinensis*

INTRODUCTION

Simmondsia chinensis (Link) Schneider (jojoba) is the sole species in the family *Simmondsiaceae* (1). It is a wind-pollinated, dioecious and a long-lived evergreen desert shrub (2, 3). Due to its economic importance, jojoba is grown in several countries such as Australia, Argentina, Chile, Egypt, South Africa and India.

Jojoba is planted commercially for its seed-liquid wax` (up 40-60 % of their dry weight); it is used as a replacement for sperm whale oil (4). Jojoba wax has potential for wider applications as extenders (5), lubricant (6), anti-foaming agents (7, 8), cosmetics and anticancer compounds and other medicinal applications such as stomachache, kidney disorders, skin disease and tending wounds (9-11). In addition, it can be used as low calorie and non-cholesterol edible oil (12).

Propagation of jojoba can be established using seeds, seedlings and stem cuttings. In jojoba as dioecious plant species, true-to-type by sexual propagation is not guaranteed. Generally, asexual propagation was established by rooting of semi-hardwood cuttings (13-15), but the maximum number of rooted cuttings is limited by plant size. Consequently, the application of micropropagaion in jojoba is essential prerequisite for commercial plantation in large scale (16). Generally, tissue culture obtained plants grow more vigorously than others obtained from seedlings and vegetative propagation where they are free from pathogens.

The role of different growth regulators and genotype in the *in vitro* propagation of jojoba was studied and a combination between the cytokinine and auxin was recommended to induce the call genesis and maximum multiplication (17, 7, 8, 18). Under these conditions, increase of seawater levels in the medium stimulated callus growth and shoot multiplication (19). These data indicated that further studies are needed to understand how jojoba micropropagation, as a stress tolerant plant species, is affected by growth regulators and how it is influenced by increasing the osmotic pressure of the medium.

Generally, it is often difficult to determine and analyze the response of specific plant species to different abiotic stresses in the field or under greenhouse conditions, due to the nature of different stresses and the difficulties to avoid completely the effects of other factors. Tissue culture techniques can establish controlled conditions and avoid the interference of other factors with the intended one; therefore, it allowed a deeper understanding of the physiology and biochemistry of plants cultured under adverse environmental conditions (20).

Gene expression studies are used to explain how plants are affected by developmental stages and/or stress agents. Previous reports suggested a great implication of specific polypeptides in plant metabolism (21). Presence or absence of isoenzyme form shows a change in the isoenzyme expression. In addition, an increase in the staining intensity of the detected band represents an increase in the activity of the studied isoenzyme (22,23) indicating that the gene involved in the synthesis of this isoenzyme form is differentially activated under the studied agent (24,23,25).

This work was fulfilled to determine the suitable growth regulator combination, which resulted in efficient *in vitro* shoot multiplication and alteration in gene expression, and how they were influenced by the osmotic potential of culture medium using different media and different concentrations of mannitol and NaCl. The effect of other stress agent such as lead acetate was also studied.

MATERIALS AND METHODS

Shoot culture establishment

Jojoba seeds were obtained from the Egyptian Natural Oil Co. S.A.E., Ismailia Farm, Salam Zone, Ismailia, Cairo, Egypt. The farm was planted in 1991 and used for research and production. Seeds were germinated in pots under culture room conditions (16 h daily light at 100 µmol m^{-2} s^{-1}, and 28 ± 2ºC) for one year. Shoots were collected and subjected for nodes sterilization in Clorox for four minutes followed by further four minutes in ethyl alcohol (70%). Then, nodal segments were washed by sterilized distilled water three times, three minutes each. Nodal segments were transferred to multiply on MS (26) medium supplemented with several concentrations of BAP (1, 2, 3 or 4 mg/l) in combination with two concentrations of NAA (0.1 or 0.2 mg/l). Cultures were incubated under culture room conditions for eight weeks.

Determination of genetic variation of *in vitro* grown jojoba plants

DNA was extracted from *in vitro* grown plants obtained from ten jojoba seeds using the modified CTAB protocol described by (27). Seven random 10-mer primers (OPA-03, OPA-04, OPA-08, OPA-13, OPC-02, OPK-02 and OPat-08) were used in this study for DNA amplification. The polymerase chain reactions (PCR) were carried out in 25 µl volume containing 50 ng of genomic DNA template, 30 pmoles/µl primers, 0.2 µM each of dATP, dCTP, dGTP and dTTP, 10 x buffer (50 mM KCl, 10 mM Tris-HCl (pH 8.3)), 2.5 mM MgCl2, and 2 units of Taq DNA polymerase (AB gene).

PCR amplification was performed in a Perkin-Elmer/Gene Amp® PCR System 9700 (PE Applied Biosystems) programmed to fulfill 35 cycles after an initial denaturation cycle for 5 min at 94ºC. Each cycle consisted of a denaturation step at 94ºC for 45 s, an annealing step at 36ºC for 50 s, and an elongation step at 72ºC for 1 min. The primer extension segment was extended to 7 min at 72ºC in the final cycle. The amplification products were resolved by electrophoresis in a 1.5% agarose gel containing ethidium bromide (0.5 µg/ml) in 1X TBE buffer at 95 volts.

Shoot multiplication and growth

To extract the best conditions suitable for jojoba microshoot multiplication and growth, the effects of the following factors were tested, where five replicates with thirty-five explants per each treatment were done, and the estimated parameters were microshoot frequency, number of

microshoots per explant, length of microshoot, number of leaves per microshoot, and number of nodes per microshoot.

Effect of different auxin/cytokinine ratios

Nodal segments of about 1.5 cm length of *in vitro* grown jojoba shoots (each contained one auxiliary bud) were cultured on MS medium supplemented with several concentrations of plant growth regulators (BAP and NAA) as indicated in table 3. Cultures were incubated under tissue culture conditions for five weeks.

Effect of GA_3 in combination with other growth regulators

Nodal segments of about 1.5 cm length of *in vitro* grown jojoba shoots were cultured on MS medium supplemented with 4 mg/l BAP, 3 mg/l BAP + 0.1 mg/l NAA with or without 0.5 mg/l GA_3 for five weeks under tissue culture conditions.

Effect of medium type on shoot multiplication and growth

Explants of about 1.5 cm length of *in vitro* grown jojoba shoots were cultured on MS, SH (28), or B5 (29) media supplemented with 3 mg/l BAP + 0.1 mg/l NAA for eight weeks and the cultures were incubated under tissue culture conditions.

Effect of NaCl, mannitol and lead acetate

Nodal segments of about 1.5 cm length of *in vitro* grown jojoba shoots were cultured on MS medium supplemented with 3 mg/l BAP + 0.1 mg/l NAA with several concentrations of NaCl (0, 0.5, 1, 2, 3 or 4 g/l), mannitol (0, 0.5, 1, 2, 3 or 4 g/l), or lead acetate (0, 4, 8, 12, 16 or 20 mg/l). Cultures were incubated under tissue culture conditions for six weeks.

Effect of growth regulators on root formation

For root induction, *in vitro* grown jojoba shoots (1.5 - 3 cm in length) were transferred to half strength MS medium supplemented with several concentrations of BAP, NAA, or IBA as included in table 9. Cultures were incubated for six weeks under tissue culture conditions.

SDS-PAGE and isoenzymes analysis

Microshoots (plant material) grown for one week under the influence of GA_3 in combination with other growth regulators, several media types, and growth regulators in combination with several concentrations of NaCl, mannitol or lead acetate were subjected for SDS-PAGE and isoenzymes analysis.

SDS-PAGE analysis

Approximately one gram of fresh plant materials was ground in a mortar and pestle in liquid nitrogen. Crashing continued until the plant materials were completely homogenized. The crushed samples were mixed with 1 ml extraction buffer (50 mM Tris-HCl buffer, pH 6.8, glycerol 10 %, 0.1 % SDS w/v and 0.3 % (v/v) 2-mercaptoethanol). The samples were subjected for centrifugation at 15000 rpm for 15 min at 4 ºC. The mixture was heated in a water bath (96 ºC) for 90 s and loaded onto gel wells for electrophoresis. Electrophoresis was performed using 10 % polyacrylamide gel at 10 ^{o}C with 2 mA per sample according to (30). Coomassie blue staining method for the SDS-PAGE was performed.

Isoenzymes' analysis

One gram of the plant materials was ground on ice in a mortar and pestle using 1 ml of 0.04 M Tris-HCl, pH 7.0, containing 2 mM cysteine. The homogenate was centrifuged at 15000 rpm for 15 min at 4 ºC. Native PAGE was performed in 7.5 % polyacrylamide slab gels. Gels were run in 0.025 M Tris-HCl + 0.192 M glycine buffer, pH 8.9, at 10 ºC with 2 mA per sample. Peroxidases were stained as described by (31).

RESULTS AND DISCUSSION

Nodal segments of one-year-old plants were sterilized as described in materials and methods section and cultured on MS medium supplemented with several concentrations of BAP and NAA (Table 1). Seven random primers were used to analyze the genomic DNA diversity among *in vitro* plants obtained from ten jojoba seeds. RAPD markers profile and their results were indicated in Fig. (1) and Table (2). The polymorphic bands percentage (number of polymorphic amplicons/total number of amplicons) for primers OPA-03, OPA-04, OPA-08, OPA-13, OPC-02, OPK-02 and OPat-08 were 43%, 88%, 83%, 62%, 75%, 75% and 75%, respectively. Consequently, primer OPA-04 was recommended to detect variation between jojoba individuals on natural grown plants as well as plants obtained from tissue culture. Since a high genetic variation was detected within the investigated plant materials, the progeny of one plant was randomly selected to carry out the other experiments.

Table 1. Induction of microshoots formation on nodal explants obtained from soil grown jojoba plants (one year old) after eight weeks of culture on MS medium supplemented with different concentrations of BAP and NAA.

Growth regulator(mg/l) BAP	NAA	Shoot frequency %	No. of shoots / explants	Length of shoot (cm)	No. of leaves / shoot	No. of nodes / shoot
1	0.1	92.85	2.6* ± 0.57	4.6* ± 1.15	6*± 0.00	5* ± 1.00
2	0.1	100	6* ± 1.00	3.5* ± 0.86	6.7 ± 0.57	4 ± 1.00
3	0.1	100	8 ± 1.00	3 ± 0.5	7 ± 1.00	4 ± 0.00
4	0.1	100	3* ± 1.00	2.9 ± 0.10	4* ± 0.00	3* ± 1.00
1	0.2	57	2* ± 0.00	3.6* ± 0.17	7 ± 1.00	3* ± 1.00
2	0.2	87.5	3* ± 0.00	2* ± 0.00	8* ± 0.00	3* ± 0.00
3	0.2	100	3* ± 0.00	1.5* ± 0.00	4* ± 0.00	2* ± 0.00
4	0.2	88.8	2* ± 0.00	2.3* ± 0.57	4* ± 0.00	2* ± 0.00

* Means significantly different (t-test) from microshoots grown on MS medium supplemented with 3 mg/l BAP + 0.1 mg/l NAA at $P < 0.05$

Table 2. RAPD markers to analyze DNA diversity among ten different *in vitro* plants obtained from ten jojoba seeds.

No	Primer	Primer sequence (5' to 3')	No. of scorable bands	Size range (bp)	Monomorphic bands	Polymorphic bands	Unique bands
1	OPA-03	AGTCAGCCAC	14	200 – 1500	7	6	1
2	OPA-04	AATCGGGCTG	9	455 – 1500	0	8	1
3	OPA-08	GTGACGTAGG	6	395 – 1305	1	5	0
4	OPA-13	CAGCACCCAC	13	200 – 1151	3	8	2
5	OPC-02	GTGAGGCGTC	8	294 – 1803	1	6	1
6	OPK-02	GTCTCCGCAA	8	370 – 2205	1	6	1
7	OPat-08	AACGGCGACA	4	430 – 1033	1	3	0

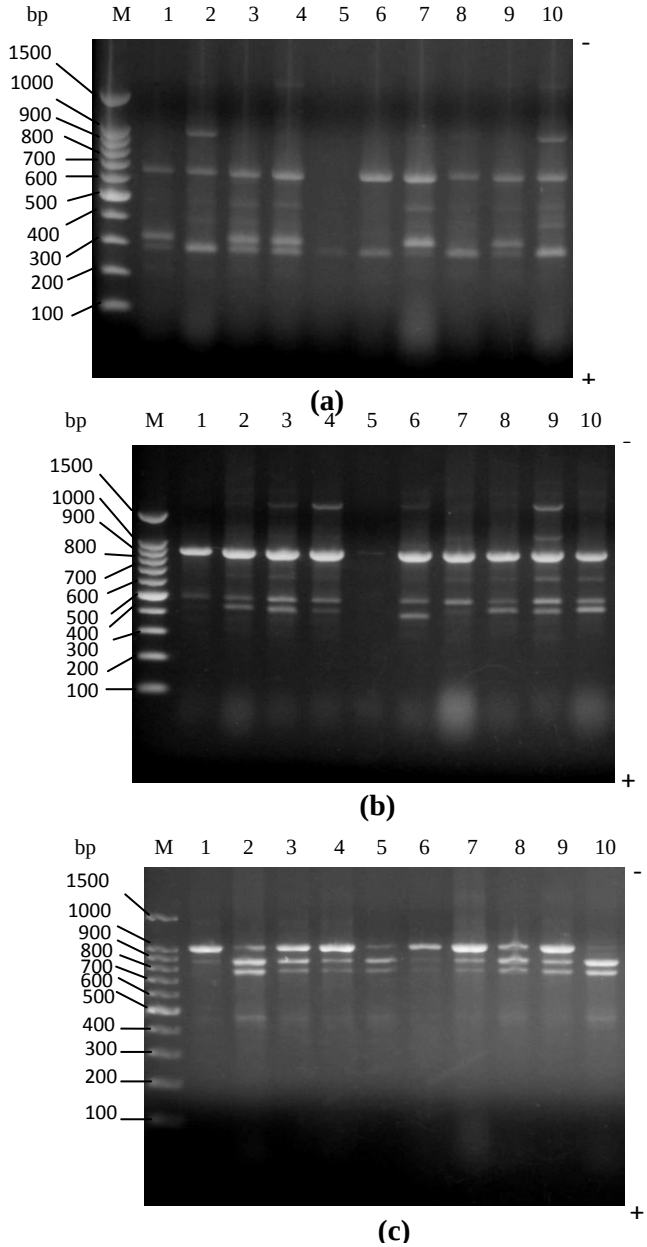


Fig. 1. RAPD profile of *in vitro* jojoba plants obtained from ten seeds using three primers: (a) OPC-02, (b) OPK-02 and (c) OPat-08

Growth regulators-free medium as well as medium containing relatively low concentrations of BAP (1 and 2 mg/l) did not show any tendency to stimulate shoot formation on the cultured explants. These concentrations of BAP showed shoot multiplication when they were used in combination with NAA. Growth regulators' combinations consisted of 2 or 3 mg/l BAP and 0.1 or 0.2 mg/l NAA resulted in high shoot multiplication, high shoot number and high growth parameters (length of shoots, number of leaves/shoot and number of nodes/shoot). The highest regeneration frequency was obtained when MS medium was supplemented with 3 mg/l BAP in combination with 0.1 mg/l NAA, thus shoots were subcultured on this type of medium to use as a source of explants in other experiments. Generally, the base of the cultured explants enlarged; this enlargement was a source for the initiation of adventitious shoots (Fig. 2).

Fig. 2. Microshoot formation on nodal explant derived from *in vitro* grown jojoba shoots after five weeks of culture on MS medium supplemented with 3 mg/l BAP + 0.1 mg/l NAA.

Two applied growth regulators were used to establish further auxin/cytokine ratios (Table 3) using *in vitro* obtained plant materials. The data indicated that 3 mg/l BAP and 0.1 mg/l NAA was still the best while decrease the concentration of BAP less than 3 mg/l decreased shoot multiplication irrespective the concentration of NAA. The values of growth parameters increased with the increase of BAP concentration. In jojoba, BAP was used in other reports and it resulted in large numbers of axillary and adventitious shoots (17). Generally, supplementing MS medium with low concentrations of NAA (0.1 or 0.2 mg/l) increased the effect of BAP on jojoba micropropagation. Consequently, MS medium supplemented with BAP in combination with NAA was better than medium with BAP alone. However, the mode of interaction between cytokinin and auxin often depends upon the plant species (32, 33).

Table 3. Effect of different concentrations of BAP and NAA on *in vitro* shoot multiplication and growth of jojoba plant after five weeks of culture.

BAP (mg/l)	NAA (mg/l)	No. of shoots/ explant	Shoot length (cm)	No. of leaves/ shoot	No of nodes/ shoot
3.75	1.3	4*± 1.0	6 ± 0.57	12 ± 2.00	7.3* ± 1.52
3.00	0.1	6 ± 2	7 ± 1.00	12 ± 2.00	8.2 ± 1.52
2.50	1.3	2* ± 0.00	4* ± 0.500	11* ± 1.00	5* ± 0.00
1.25	1.3	2* ± 0.00	3.5* ± 0.00	7.3* ± 1.15	4.6* ± 0.57
1.25	2.6	1* ± 0.00	4* ± 0.50	8* ± 0.00	4.3* ± 0.57
1.25	3.9	1* ± 0.00	2.8* ± 0.20	6* ± 2.00	4* ± 1.00

* Means significantly different (t-test) from jojoba microshoots grown on MS medium supplemented with 3 mg/l BAP + 0.1 mg/l NAA at $P < 0.05$.

Growth regulators combinations including GA_3 were applied to investigate the effect of GA_3 on shoot multiplication and growth (Table 4). While gibberellins stimulate cell elongation and other physiological processes, GA_3 expressed a negative effect on jojoba shoot multiplication and growth. Reduction in shoot multiplication and shoot growth was recorded when jojoba shoots were cultured on MS medium supplemented with 0.5 mg/l GA_3 alone or in combination with BAP and/or NAA. (16) used MS medium supplemented with 1 mg/l BAP and 0.5 mg/l GA_3 to enhance bud break frequency but one shoot/node was only formed. Our work indicated that, application of GA_3 is not recommended for *in vitro* multiplication of jojoba.

Table 4. Effect of combinations of different plant growth regulator on *in vitro* multiplication and growth of jojoba plant after five weeks of culture.

BAP (mg/l)	NAA (mg/l)	GA_3 (mg/l)	No. of shoots/ explants	Shoot length (cm)	No. of leaves/ shoot	No. of nodes/ shoot	Shoot fresh weight (g)
--	--	--	2 ± 0.00	4.5 ± 0.50	11 ± 1.00	6 ± 1.00	0.20 ± 0.00
4	--	--	3* ± 0.00	7* ± 2.00	14* ± 0.00	8* ± 1.00	0.16 ± 0.01
3	0.1	--	6* ± 2.00	5* ± 1.00	17* ± 0.00	7* ± 0.00	0.20 ± 0.02
--	--	0.5	1* ± 0.00	3.5* ± 0.50	9* ± 1.00	4.5* ± 0.50	0.16 ± 0.04
4	--	0.5	2 ± 0.00	4.5 ± 0.50	10* ± 0.00	5* ± 0.00	0.17 ± 0.02
3	0.1	0.5	3* ± 1.00	4* ± 0.50	11 ± 1.00	5* ± 0.00	0.18 ± 0.04

* Means significantly different (t-test) from jojoba microshoots grown on MS medium without growth regulators at $P < 0.05$.

The effect of the selected growth regulators combination (3 mg/l BAP + 0.1 mg/l NAA) on jojoba multiplication and growth depended on the type of the used basal medium (Table 5). Media types have different osmotic potentials due to

their different chemical components. Some of them were described as high salt levels such as MS and SH, where MS contains the highest concentrations of minerals (26). While all the tested media were supplemented with 3 mg/l BAP and 0.1 mg/l NAA, they expressed different effects on jojoba multiplication and growth. It is well known that, nutritional requirements for optimal growth of the cultured explants varied with species and it played a key role in morphogenesis and response of explants (34-36). In our work, in eight weeks, on different regeneration media (MS, SH and B5), MS medium was the best where it resulted in the highest shoot number/explant and the highest growth parameters including shoot length, number of leaves and nodes/shoot. On the other side, the lowest salt medium, Gamborg's B5 (29), expressed the lowest number of formed shoots as well as shoot growth parameters. It was clear that increasing the osmotic potential of the medium improved *in vitro* multiplication of jojoba as a stress tolerant plant species. Therefore, Gamborg's B5 medium was not suitable for jojoba micropropagation because it has lower osmotic potential and nitrogen content than Murashige and Skoog (MS) medium.

Table 5. Effect of basal medium type on *in vitro* multiplication and growth of jojoba plant after eight weeks of culture on the basal medium type supplemented with 3 mg/l BAP + 0.1 mg/l NAA.

Type of medium	No. of shoots / explant	Shoot length (cm.)	No. of leaves / shoot	No. of nodes / shoot
MS	8 ± 2	8.6 ± 0.51	14.3 ± 2.51	8.6 ± 0.52
SH	3* ± 1.00	7.6* ± 0.76	13.3* ± 1.15	8 ± 1.70
B5	2* ± 1.00	6.2* ± 1.60	10.6* ± 1.15	6.3* ± 0.57

* Means significantly different (t-test) from jojoba microshoots grown on MS medium supplemented with 3 mg/l BAP + 0.1 mg/l NAA at $P < 0.05$.

The effect of the selected growth regulators (3 mg/l BAP + 0.1 mg/l NAA) combination on jojoba shoot multiplication and growth was magnified by the addition of NaCl or mannitol (Table 6 and 7), especially in low concentrations (0.5 g/l). It may be due to the increase in the osmotic potential of the cultured medium. Shoot multiplication was more sensitive for NaCl than shoot growth. Where, jojoba growth parameters were improved under the influence of NaCl or mannitol up to 3 g/l, but the relatively high concentration (4 g/l) had a negative effect, where all the determined parameters were decreased (Table 6 and 7). Shoot multiplication was improved only under the influence of 0.5 mg/l NaCl. (37) found that the application of seawater in low levels did not inhibit growth of jojoba shoots. On the other side, shoot multiplication and growth were negatively influenced under the effect of lead acetate even in low concentrations (Table 8). It may be due to reducing the uptake and transport of nutrients such as Ca,

Fe, Mg, Mn, P, and Zn, or negative interference with several physiological and biochemical processes (38).

Table 6. Effect of various concentrations of NaCl with MS medium + 3 mg/l BAP + 0.1 mg/l NAA, on *in vitro* shoot multiplication and growth of jojoba plant after six weeks of culture.

NaCl (g/l)	No. of shoots / explant	Shoot length (cm)	No. of leaves / shoot	No. of nodes/ shoot	Shoot fresh weight (g)
0	8 ± 2.00	11 ± 0.50	14 ± 2.00	8 ± 1.00	0.20 ± 0.02
0.5	12* ± 0.00	11.5 ± 0.50	16* ± 2.00	8 ± 1.00	0.23 ± 0.01
1	6* ± 1.00	13.3* ± 0.57	16.6* ± 1.15	9* ± 1.00	0.22 ± 0.01
2	3* ± 1.00	12.3* ± 0.57	16* ± 2.00	9* ± 1.00	0.22 ± 0.02
3	2* ± 1.00	12* ± 0.50	19* ± 1.00	7* ± 1.00	0.20 ± 0.01
4	2* ± 1.00	9.5* ± 0.50	14 ± 2.00	7* ± 1.73	0.16 ± 0.01

* Means significantly different (t-test) from jojoba microshoots grown on MS medium supplemented with 3 mg/l BAP + 0.1 mg/l NAA without NaCl at $P < 0.05$.

Table 7. Effect of various concentrations of mannitol in MS medium + 3 mg/l BAP + 0.1 mg/l NAA on *in vitro* shoot multiplication and growth of jojoba plant after six weeks of culture.

Mannitol (g/l)	No. of Shoots/ explant	Shoot length (cm)	No. of leaves/ shoot	No. of node/ shoot	Shoot fresh weight (g)
0	8 ± 2.00	11 ± 0.50	16 ± 2.00	8 ± 1.00	0.26 ± 0.01
0.5	10*± 2.64	11 ± 0.50	17* ± 1.00	8 ± 1.00	0.28 ± 0.01
1	8 ± 2.64	12* ± 1.00	16 ± 1.00	8 ± 1.00	0.28 ± 0.01
2	4* ± 1.73	12.5* ± 0.50	14* ± 2.00	7* ± 1.73	0.27 ± 0.03
3	1* ± 0.00	11.5 ± 0.86	12* ± 2.00	7* ± 1.73	0.28 ± 0.01
4	1* ± 0.00	10.5 ± 0.50	12* ± 3.46	8 ± 1.73	0.27 ± 0.01

* Means significantly different (t-test) from jojoba microshoots grown on MS medium supplemented with 3 mg/l BAP + 0.1 mg/l NAA without mannitol at $P < 0.05$.

Table 8. Effect of various concentrations of lead acetate in MS medium + 3 mg/l BAP + 0.1 mg/l NAA on *in vitro* multiplication and growth of jojoba plan after six weeks of culture.

Lead acetate (mg/l)	No. of shoots/ explants	Shoot length (cm)	No. of leaves/ shoot	No. of nodes/ shoot	Shoot fresh weight (g)
0	10 ± 1.00	12. ± 0.76	16 ± 0.00	8 ± 1.00	0.22 ± 0.08
4	3* ± 1.00	5.5* ± 0.00	12* ± 0.00	6* ± 0.00	0.19 ± 0.09
8	3* ± 1.00	6.5* ± 0.00	11* ± 0.00	6* ± 1.00	0.19 ± 0.00
12	2* ± 1.00	5.5* ± 0.00	10* ± 0.00	5* ± 0.00	0.18 ± 0.08
16	2* ± 1.00	5.5* ± 0.50	15* ± 0.00	7* ± 0.00	0.19 ± 0.00
20	1* ± 0.00	5* ± 1.00	10* ± 1.00	7* ± 0.00	0.10 ± 0.00

* Means significantly different (t-test) from jojoba microshoots grown on MS medium supplemented with 3 mg/l BAP + 0.1 mg/l NAA without lead acetate at $P < 0.05$.

For root formation, microshoots were transferred to half strength MS medium supplemented with several concentrations of BAP, NAA, and IBA. After six weeks, the best root frequency (57%) was achieved on the medium supplemented with 1.25 mg/l BAP + 1.3 mg/l NAA (Table 9). On the other hand, the highest root length was recorded when jojoba microshoots were cultured on the medium supplemented with 5 mg/l IBA alone. In addition, the lowest number and length of roots was recorded when NAA concentration was increased to 2.6 mg/l in combination with BAP (1.25 mg/l) and IBA (2 mg/l). Rooted jojoba miroshoots were transferred to MS medium without growth regulators for further three weeks, where root number and length increased (Fig. 3). Auxins alone or in combination with other hormones were used for induction of roots formation (39,8,40). Rooted plantlets were transferred to grow under greenhouse conditions after acclimatization.

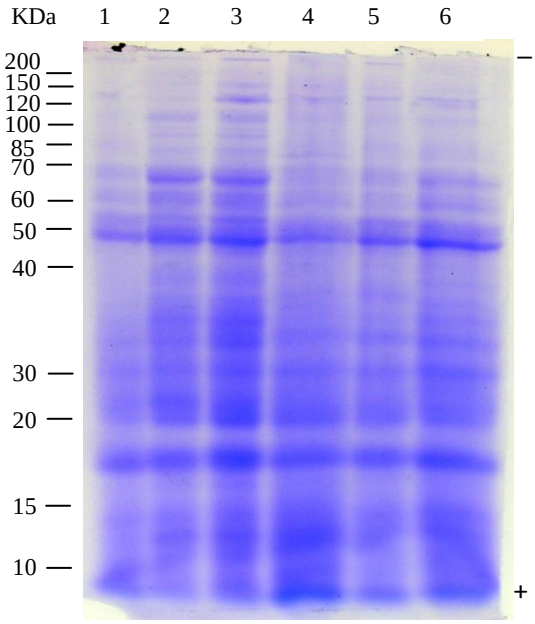
Table 9. Induction of adventitious root formation on jojoba microshoots as influenced by half strength MS medium supplemented with different growth regulators for six weeks.

Phytohormone (mg/l)			Rooting (%)	No. of roots/ shoot	Root length (cm)
BAP	**NAA**	**IBA**			
1.25	1.3	--	57	3 ± 1.00	3.5 ± 0.50
1.25	2.6	--	30	2* ± 1.00	1.75* ± 0.25
3.75	1.3	--	10	3 ± 1.00	0.7* ± 0.30
1.25	2.6	2	20	1.3* ± 0.57	1* ± 0.00
1.25	1.3	2	40	3.3 ± 1.52	2.25* ± 0.25
--	--	5	10	2* ± 0.00	5* ± 1.73

* Means significantly different (t-test) from jojoba microshoots grown on half strength MS medium supplemented with 1.25 mg/l BAP + 1.3 mg/l NAA at $P < 0.05$.

Fig. 3. Root formation on jojoba microshoots after six weeks of culture on root induction medium followed by three weeks on MS medium without growth regulators.

Comparison between SDS-PAGE pattern of jojoba shoot cuttings (Fig. 4) grown on growth regulators-free medium and others grown on BAP alone or in combination with NAA indicated that conditions favored jojoba micropropagation, favored also gene expression where new polypeptide bands were detected and the staining intensity of some bands was increased. On the other hand, the lowest staining intensity of polypeptide bands was detected when shoot cuttings were cultured under jojoba micropropagtion unsuitable conditions; on growth regulators-free MS medium (lane 1) or on MS containing GA_3 alone (lane 4). The staining intensity of bands increased when GA_3 was used in combination with other growth regulators (lane 6), where, the negative effect of GA_3 on shoot multiplication and growth was improved when GA_3 was used in combination with BAP and NAA. In addition, four new polypeptides (90, 80, 39 and 25.6 KDa) were detected when the jojoba microshoots expressed shoot multiplication on MS medium supplemented with 4 mg/l BAP (Lane 2). Further four polypeptide bands (151.4, 150, 72.5 and 16.2 KDa) were detected when the medium contained growth regulators favor maximum shoot multiplication and growth (3 mg/l BAP + 0.1 mg/l NAA. On the other hand, one polypeptide band with molecular weight of 81.4 KDa appeared when jojoba microshoots were subcultured on MS medium supplemented with 0.5 mg/l GA_3; this polypeptide band did not appeared in other treatments. Peroxidase isoenzyme



pattern was in accordance with SDS-PAGE and showed the appearance of new isoenzyme form (POX-1) and increase in the staining intensity of the detected bands when the medium containing growth regulators stimulated shoot multiplication and shoot growth (Fig. 5).

Fig. 4. SDS-PAGE of *in vitro* grown jojoba shoot cuttings after one week of culture on MS medium (lane 1) supplemented with 4 mg/l BAP (lane 2); 3 mg/ l BAP + 0.1 mg/l NAA (lane 3); 0.5 mg/l GA3 (lane 4); 4 mg/l BAP + 0.5 mg/l GA3 (lane 5); and 3 mg/l BAP + 0.1 mg/l NAA + 0.5 mg/l GA3 (lane 6)

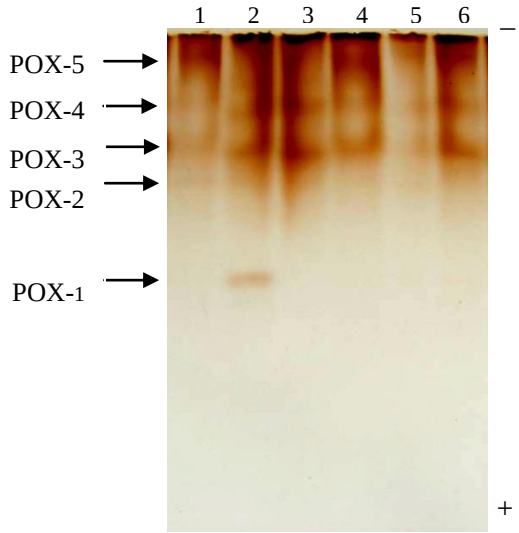


Fig. 5. Native gel electrophoresis of POX isoenzyme pattern of *in vitro* grown jojoba shoot cuttings after one week of culture on MS medium (lane 1) supplemented with 4 mg/l BAP (lane 2); 3 mg/l BAP + 0.1 mg/l NAA (lane 3); 0.5 mg/l GA3 (lane 4); MS with 4 mg/l BAP + 0.5 mg/l GA3 (lane 5); and 3 mg/ l BAP + 0.1 mg/l NAA + 0.5 mg/l GA3 (lane 6).

Jojoba explants cultured on different regeneration media with 3 mg/l BAP + 0.1 mg/l NAA (Fig. 6) showed different protein expressions. The best basal medium, MS, also expressed the highest staining intensity and polypeptide number. Several polypeptide bands which appeared under the influence of conditions stimulated the best shoot multiplication and growth (151.4, 150, 39, 25.6 and 16.2 KDa), disappeared under the influence of other media especially B5 medium where shoot multiplication and growth were the worst. On the other side, conditions retarded jojoba micropropagation, on B5 medium, resulted in the appearance of three polypeptide bands (204, 85 and 55.7 KDa). In addition, conditions favored shoot multiplication on MS and SH media resulted in expression of POX-1 and increased the staining intensity of the detected isoenzyme bands (Fig. 7). Isoenzyme form marked "POX-1" was not detected when jojoba shoots were subcultured on B5 medium (lane 3).

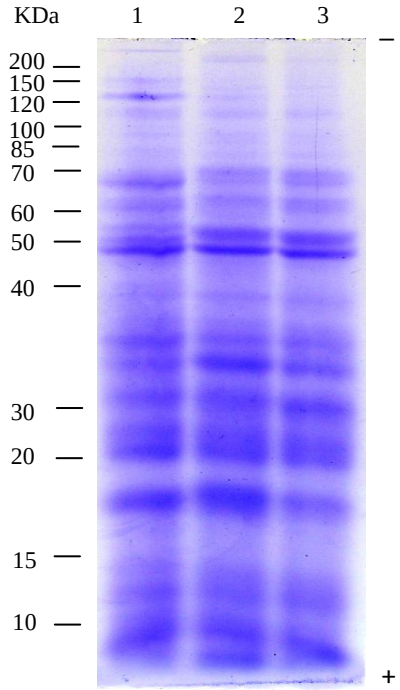


Fig. 6. SDS-PAGE of *in vitro* grown jojoba shoot cuttings after one week of culture on MS, SH, or B5 media (lanes 1, 2 or 3 respectively). Media were supplemented with 3 mg/l BAP + 0.1 mg/l NAA.

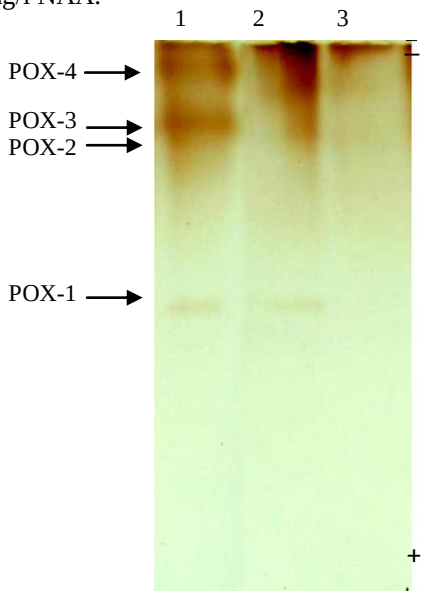


Fig. 7. Native gel electrophoresis of POX isoenzyme pattern of *in vitro* grown jojoba shoot cuttings after one week of culture on MS, SH, or B5 media (lanes 1, 2 or 3, respectively). Media were supplemented with 3 mg/l BAP + 0.1 mg/l NAA.

Conditions stimulated shoot growth on MS medium supplemented with 3 mg/l BAP + 0.1 mg/l NAA and NaCl up to 3 g/l resulted in the highest number of the detected polypeptides (Fig. 8), where seven polypeptide bands with molecular weights of 135.3, 124.6, 113, 85, 36.26, 35.2 and 12.3 KDa were detected. These bands disappeared when microshoot cuttings were subcultured on the medium containing 4 g/l NaCl. In addition, 4 polypeptide bands with molecular weights of 90, 80, 39 and 15 KDa disappeared but one with molecular weight of 110 was detected when microshoot cuttings were subjected to the medium containing 4 g/l NaCl (lane 6). Under stress condition, the function of the newly formed proteins is to play an important role in stress tolerance (41) through protecting the integrity of cell membranes and their macromolecules, binding to excess salts, reducing toxicity, scavenging of free radicals and offsetting of salt-inactivated proteins (42). Many reports indicated that protein pattern changes are in accordance with biological adaptation processes, which make the organism more fit to the established environment (43). In accordance with SDS-PAGEs, peroxidase isoenzyme patterns showed the appearance of new isoenzyme forms and increase the staining intensity of the detected bands when the applied conditions stimulated shoot multiplication and shoot growth. This enzyme system has been used as a model to study hormonal control of growth and morphogenesis processes in plants (44). The effect of mannitol was similar to that of NaCl, where bands with molecular weights of 135.3, 124.6, 85, and 77 KDa were detected when mannitol concentrations did not retard shoot growth, these conditions resulted also in increase of peroxidase expression (data not shown).

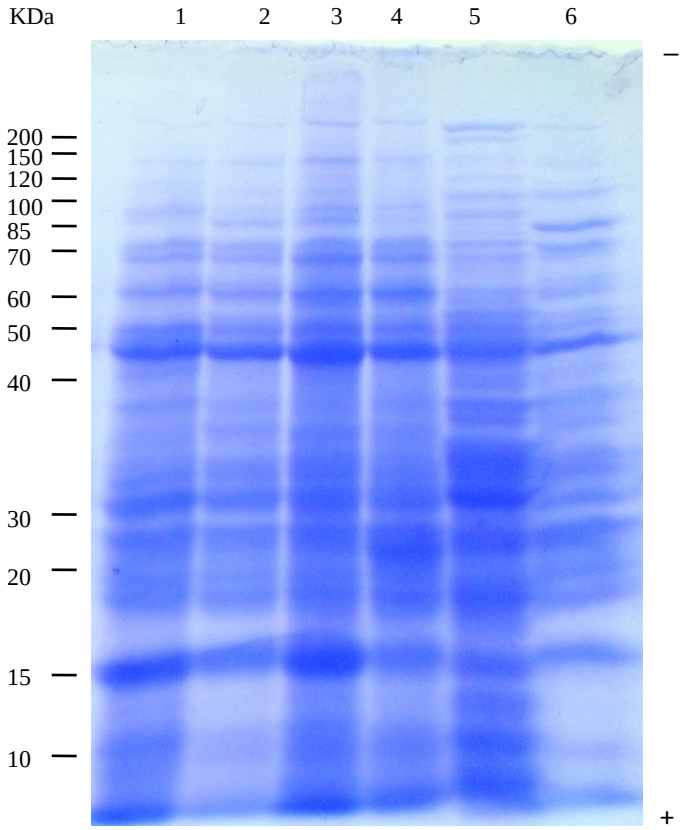


Fig. 8. SDS-PAGE of *in vitro* grown jojoba shoot cuttings after one week of culture on MS medium supplemented with 3 mg/l BAP + 0.1 mg/l NAA and 0, 0.5, 1, 2, 3 or 4 g/l NaCl (lanes 1, 2, 3, 4, 5 or 6, respectively).

SDS-PAGE pattern of jojoba shoot cuttings under the influence of different concentrations of lead acetate was studied (Fig. 9). In general, stability of the protein pattern was main texture phenomenon with tendency to increase the staining intensity of several polypeptide bands. Under relatively high concentrations of lead acetate (0.016 and 0.02 g/l), polypeptides with molecular weights of 100 and 115 KDa were detected but two others disappeared (80 and 90 KDa). The number of isoperoxidases and staining intensity of the detected bands increased when jojoba shoots were cultured on MS medium supplemented with 3 mg/l BAP + 0.1 mg/l NAA and several concentrations of lead acetate. Two isoenzyme forms (POX-1 and POX-2) were detected and the staining intensity of all the detected bands was progressively increased with the increase of lead in the cultured medium (Fig. 10). Peroxidase included strongly in shoot differentiation and adaptation of the cultured tissues to specific stress agent where the regeneration potential of the cultured tissue was dependent on both scavenging potential and the

ability to produce H_2O_2 through NADH-POX activity, which control shoot multiplication and growth under the applied conditions (45).

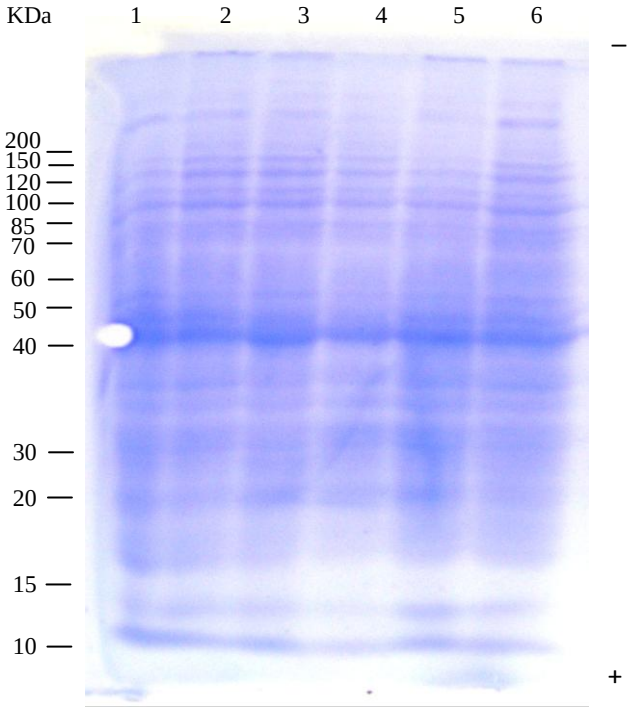


Fig. 9. SDS-PAGE of *in vitro* grown jojoba shoot cuttings after one week of culture on MS medium supplemented with 3 mg/l BAP + 0.1 mg/l NAA and 0, 0.004, 0.008, 0.012, 0.016 or 0.02 g/l lead acetate (lanes 1, 2, 3, 4, 5 or 6, respectively).

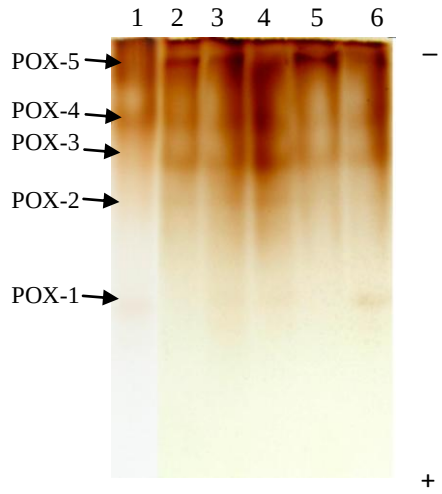


Fig. 10. Native gel electrophoresis of POX isoenzyme pattern of *in vitro* grown jojoba shoot cuttings after one week of culture on MS medium supplemented with 3 mg/l BAP + 0.1 mg/l NAA and 0, 0.004, 0.008, 0.012, 0.016 or 0.02 g/l lead acetate (lanes 1, 2, 3, 4, 5 or 6, respectively).

In conclusion, as jojoba is a stress tolerant species, a right combination of growth regulators should be used for *in vitro* shoot multiplication. To improve the data, increase of the osmotic potential of the cultured medium using high salt medium such as MS medium supplemented with relatively low concentration of NaCl or mannitol was recommended. Further studies should be done on other salt tolerant plant species to know if it is a common phenomenon or not.

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تحسين الإكثار المعملي لنبات الجوجوبا باستخدام تركيزات منخفضة من الملح أو المانيتول

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كلية العلوم

تمت هذه الدراسة لفهم تأثير بعض العوامل علي الإكثار المعملي لنبات الجوجوبا مثل منظمات النمو وكذلك التعبير الجيني للنبات تحت ظروف الإجهاد المختلفة. تم دراسة التغيرات الوراثية لنبات الجوجوبا وذلك باستخدام أفراد ناتجة من انبات بذور مختلفة باستخدام تقنية الرابد ووجد أن هناك اختلافات وراثية شديدة فيما بينها، ولذلك تمت كل تجارب هذه الدراسة علي نبات واحد فقط من تلك النباتات لضمان ثبات المصدر الجيني. وجد أن تركيز ٣ مجم/لتر من البنزيل أمينو بيورين مع ٠.١ مجم/لتر من نفثالين حمض الخليك هما أفضل تركيزان لإكثار نبات الجوجوبا معملياً ولذلك توصي الدراسة بهما، بينما نقص تركيز البنزيل أمينو بيورين وزيادة تركيز نفثالين حمض الخليك عن ذلك أدي إلي إنخفاض تضاعف الأفرع الخضرية للنبات ونموها. أوضحت الدراسة أن حمض الجبريلليك يثبط الإكثار المعملي للجوجوبا ولذلك لا توصي الدراسة باستخدامه. في نفس الوقت وجد أن استخدام تركيزات منخفضة من أي من كلوريد الصوديوم أو المانيتول مع نسبتي البنزيل أمينو بيورين ونفثالين حمض الخليك الموصي بهما حسنت تضاعف الأفرع الخضرية ونموها عند اضافتهما للوسط الغذائي موراشيجي وسكوج والذي يعتبر من الأوساط الغذائية عالية تركيز الأملاح. استخدام كلوريد الصوديوم أو المانيتول بتركيزات عالية نسبياً (٤ جم/لتر) أثر سلباً علي إكثار النبات معملياً. علي الجانب الآخر وجد أن استخدام خلات الرصاص حتي عند التركيزات المنخفضة أدي إلي إجهاض التأثير المحفز لكل من البنزيل أمينو بيورين وثفثالين حمض الخليك بالنسب الموصي بها. وجدت الدراسة أن تأثير منظمات النمو وعوامل الإجهاد المختلفة كان مصحوباً بتغيرات في التعبير الجيني للنبات وذلك من خلال تتبع دراسة الأيزوإنزيمات والبروتينات المدنترة باستخدام الفصل الكهربي (الإليكتروفوريسيز).