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An Improved Regeneration Protocol of Female Jojoba (*Simmondsia chinensis*) Plants from Callus Derived from Leaf Explants

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Abstract: The current work was conducted to improve the regeneration capacity of jojoba plants from callus derived from female leaf explants. Therefore, an improved culture medium was developed and used in the main experiments of regeneration *via* induction of adventitious shoot buds. Thus, leaf explants of female jojoba plants were cultured on an improved media for regeneration of jojoba plants; callus induction medium (CIM), bud formation medium (BFM), shoot development medium (SDM) and root development medium (RDM), containing various concentrations of plant growth regulators, auxin (2, 4-D, Dicamba, NAA or IBA) either alone or in combination with cytokinin (BA or TDZ). The obtained results revealed that, the use of the combination between Dicamba (1.5 mg/l) and TDZ (0.75 mg/l) in CIM produced the highest percentage of callus induction (98.65 %). In addition, the use of TDZ (2.5 mg/l) in combination with Dicamba (0.5 mg/l) in BFM produced the highest percentage of shoot regeneration (99.65%) with the highest average number of the produced shoots/callus (23.18). The results of root formation elucidated that, the inclusion of BA (1 mg/l) in combination with (IBA 1.5 mg/l + NAA 1.5 mg/l) led to a significant increase in the percentage of shoots produced roots and recorded the highest level (100.00 %) and the highest average number of regenerated roots/plantlet (7.11) compared to the combination between (IBA 1.5 mg/l + NAA 1.5 mg/l) which recorded (58.13 %) and (4.38 roots/plantlet). Accordingly, the average number of regenerated roots/plantlet was positively correlated with the percentage of the survival rate of the acclimatized jojoba plants, whereas the highest survival rate (96.41 %) was recorded for the plants with the highest average number of regenerated roots/plantlet (7.11). In addition to the use of the nutrient solution 1/4 strength of MS medium for irrigation of jojoba plants and the daily foliar application of plants with 1/10 strength of MS supplemented with BA (1 mg/l) + TDZ (1 mg/l) + GA3 (0.5 mg/l) during the acclimatization period (10 weeks) which proved to be essential to obtain the highest percentage of the survival rate of the acclimatized jojoba plants. The obtained results in this study proved that, a highly efficient regeneration protocol of female jojoba plants *via* adventitious shoot bud formation from callus derived from female leaf explants with the highest regeneration capacity of jojoba plants (99.65 %) and the highest average number of the produced shoots/callus (23.18) was achieved. Furthermore, this improved protocol of jojoba regeneration from leaf explants *via* adventitious shoot bud formation is of great importance for establishing a reliable protocol for *Agrobacterium tumefaciens*-mediated transformation of jojoba plants.

Key words: Callus · Regeneration · Adventitious shoot · *Simmondsia chinensis* · Jojoba

INTRODUCTION

Jojoba plant [*Simmondsia chinensis* (Link) Schneider] is a perennial evergreen, cross-pollinated desert shrub and has a long life (100- 200 years) belongs to the family *Simmondsiaceae* and is native to the Sonora desert which located between the southwest of the USA and the northwest of Mexico [1]. Jojoba is an important plant has a high economic potential due to the presence of a liquid wax in the seeds which representing about (50-55%) of the seed weight. In addition, the properties of jojoba oil are similar to the oil of the sperm whale [2]. Therefore, jojoba oil and its derivatives were used widely in the industry for producing pharmaceutical and medicinal products in

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addition to cosmetics such as (face creams, lipsticks, skin fresheners soaps and shampoos), antifoaming agents and resistant lubricants to high temperature and pressure [3]. Jojoba is a dioecious and the propagation of this plant is mainly using mature seeds which produce high variation among the trees in seed yield and seed oil content. Moreover, propagation of jojoba using mature seeds confronted with a serious problem due to the high ratio of male bias (5:1) male: female, respectively, in addition to the flowering and seed set occurs after 3-4 years of plant sowing [4, 5]. Therefore, regeneration of jojoba female plants through the production of multiple shoots from nodal explants is the current *in vitro* culture protocol for jojoba micropropagation [6-8].

So far, the available studies on indirect regeneration of jojoba from callus either *via* embryogenesis or organogenesis proved that jojoba is a recalcitrant plant, thus, only one report on somatic embryogenesis using callus derived from leaf explants was published by Hamama *et al.* [9] and only two reports on somatic embryogenesis using callus derived from immature embryos of jojoba according to Gaber *et al.* and Mohammed *et al.* [10, 11], as well as, only one report was published on organogenesis using callus derived from leaf explants [12]. Moreover, until now no reports were published on successful genetic transformation of jojoba due to the difficulties present in jojoba regeneration from callus tissue even derived from immature embryos, consequently, the establishment of regeneration protocol for jojoba from callus derived from leaf explants is still a challenge and need to be more reproducible in different laboratories to achieve a real progress in developing a genetic transformation protocol of jojoba which is essential for improving jojoba plants through genetic engineering.

Therefore, the present work was conducted aiming to achieve an efficient protocol of jojoba regeneration from callus derived from female leaf explants as a prerequisite step for establishing a reliable protocol for *Agrobacterium tumefaciens*-mediated transformation of jojoba plants. Accordingly, at the beginning the published regeneration protocols of jojoba were precisely examined in our laboratory whereas, a set of preliminary experiments were carried out to evaluate previous work on *in vitro* culture of jojoba and a low regeneration capacity of jojoba was obtained. Therefore, another set of preliminary experiments were conducted to determine the effect of media composition and the concentrations of various plant growth regulators on callus induction from female leaves of jojoba and the regeneration of shoots from callus. Thus, an improved culture medium for induction of adventitious shoot buds from callus derived from leaves of female jojoba plants was developed and used in the main experiments of this study, in addition to various combinations of plant growth regulators.

MATERIALS AND METHODS

Composition of Culture Media: In this work an improved culture medium for induction of adventitious shoot buds from callus derived from leaves of female jojoba plants was used as shown in (Table 1), this medium was developed through intensive studies on the effect of media composition on shoot induction from jojoba leaf segments, in addition to the growth and development of the produced jojoba shoots. Therefore, various jojoba media with different concentrations of plant growth regulators were used; callus induction medium (CIM), bud formation medium (BFM), shoot development medium (SDM) and root development medium (RDM), using the improved medium (a) which differ than the composition of MS medium (b) as shown in Table 1. Accordingly, the improved culture medium containing low levels of ammonium (in CIM; 14.63 mM, in BFM and SDM; 2 mM and in RDM; 7.31 mM) compared to the MS medium according to Murashige and Skoog [13] which contains (20.63 mM). In addition to the reduced levels of chloride (in CIM, BFM and SDM; 5.99 µM and in RDM; 2.99 µM) in comparison to 5.99 mM in MS medium due to the presence of calcium chloride ($CaCl_2.2H_2O$) 444 mg/l. While the level of copper was elevated to 5 µM compared to 0.1 µM in MS medium. Moreover, silver nitrate (5.9 µM), Casein hydrolysate 300 mg/l, myo-inositol 250 mg/l, B5 vitamins according to Gamborg *et al.* [14] and additional vitamins were added to this improved medium, in addition to Spermidine (1 mg/l) and adenine sulphate 50 mg/l in (BFM and SDM) as shown in (Table 1). The culture media were prepared using MS salts as concentrated individual stocks; (20 x) of each macronutrient stock and (100 x) of each micronutrient stock, sucrose, plant growth regulators and vitamins stock in addition to casein enzymatic hydrolysate, myo inositol and freshly prepared $AgNO_3$ as silver thiosulfate (STS); 1 mg/l $AgNO_3$ + 3.716 mg/l sodium thiosulfate anhydrous. The prepared solution of each culture medium 500 ml (2x) used in this study was filter-sterilized through a 0.22 µm Durapore PVDF filter WHPL 47 mm (Millipore Cat. No. GVWP04700), then mixed with 500 ml of Gelrite® (2x) which was sterilized for 20 min by an autoclave at (121°C and 15 psi) and divided into 50 ml in Petri dishes or glass jars (volume 400 ml) or magenta boxes.

Table 1: Composition of various jojoba media; callus induction medium (CIM), bud formation medium (BFM), shoot development medium (SDM) and root development medium (RDM), using the improved medium (a) compared to the composition of MS medium (b)

		Improved medium (a)			
	MS medium (b) mg/l	CIM mg/l	BFM mg/l	SDM mg/l	RDM mg/l
Salts					
*Salts MS	2, 543.31	2, 543.31	2, 543.31	2, 543.31	1, 271.65
$CaCl_2.2H_2O$	440	0.44	0.44	0.44	0.22
NH_4NO_3	1650	1170	165	165	585
$Ca(NO3)_2.4H_2O$	-	706.8	706.8	706.8	353.4
$CuSO_4 .5H_2O$	0.025	1.25	1.25	1.25	0.625
*$AgNO_3$	-	1	1	1	1
Amino acids					
Casein hydrolysate	-	300	300	300	300
Glycine	2	-	-	-	-
Vitamins					
Thiamine.HCl	0.1	10	10	10	10
Pyridoxine.HCl	0.5	1	1	1	1
Nicotinic acid	0.5	1	1	1	1
Pantothenate	-	0.5	0.5	0.5	0.5
Biotin	-	0.01	0.01	0.01	0.01
Riboflavin	-	0.01	0.01	0.01	0.01
Folic acid	-	0.01	0.01	0.01	0.01
Ascorbic acid	-	100	100	100	100
Myo-inositol	100	250	250	250	250
Spermidine	-	1	1	1	-
Adenine sulphate	-	-	50	50	-
Sucrose	30, 000	40, 000	20, 000	20, 000	20, 000
Gelrite	3, 000	2, 500	2, 500	2, 500	2, 000
pH	5.8	5.8	5.8	5.8	5.8

*Salts MS; salts of MS without (NH_4NO_3, $CaCl_2.2H_2O$ and $CuSO_4 .5H_2O$). *$AgNO_3$ was freshly prepared as silver thiosulfate (STS): 1 mg/l $AgNO_3$ + 3.716 mg/l sodium thiosulfate anhydrous

Sterilization of Plant Material: Young shoots of jojoba female plants 7 years old grown in the field of the experimental research unit of Plant Biotechnology Research Laboratories (PBRL), Plant Physiology Department, Faculty of Agriculture, Cairo University, Egypt, were used. The collected shoots were washed with running tap water for 30 min then soaked in tap water with liquid detergent for 30 min then washed with running tap water for 10 min followed by 3 times with sterilized distilled water in the laminar air flow hood. Afterwards, the shoots were surface sterilized with 70 % ethanol (v/v) for 1 min then washed one time with sterilized distilled water, followed by 0.1 % $HgCl_2$ (w/v) with 0.05 % Tween-20 for 5 min then rinsed 5 times with sterilized distilled water.

Culture of Jojoba Leaf Explants for Callus Induction: The leaves were removed from the explants (each explant contain the shoot apex followed by two nodes including 4 young leaves) then each leaf was cut into segments about 1 cm^2 then cultured with the adaxial side down onto callus induction medium (CIM) in Petri dishes and kept at 24°C in the light (16 hr /8 hr light/dark). The leaf explants were cultured for 8 weeks (subcultured every 2 weeks on a fresh CIM).

Regeneration of Jojoba Plants *via* Adventitious Bud Formation: After 8 weeks the induced calli were transferred to bud formation medium (BFM) for 10 weeks (subcultured every 2 weeks). The produced shoot buds were transferred to shoot development medium (SDM) for 10 weeks (subcultured every 2 weeks), then the regenerated shoots in length about 3 cm were transferred to root development medium (RDM) for 6 weeks (subcultured every 3 weeks). The regenerated jojoba plantlets with strong roots were transferred to small pots filled with a mixture of peat moss and fine sand (3:1) and kept for acclimatization in a growth chamber at 22°C and 85 % humidity.

Acclimatization Stage of the Regenerated Jojoba Plants: The acclimatization of the regenerated jojoba plants was conducted on two steps; the first step was in a growth

chamber for 6 weeks under temperature started with 22°C and increased 2°C every week up to 32°C in the sixth week and with humidity started with 85 % and decreased gradually 5 % every week to 60 % in the sixth week, then the second step was in the greenhouse for 4 weeks, thus the plants were transferred to big pots (30 cm in diameter) filled with a mixture of peat moss and fine sand (3:1) and grown under 35°C and about 55 % humidity. During the acclimatization stage, the plants were irrigated with a nutrient solution (1/4 strength of MS salts) and daily sprayed with a nutrient solution (1/10 strength of MS salts) supplemented with (BA 1 mg/l + TDZ 1 mg/l + GA3 0.5 mg/l).

Statistical Analysis: The obtained data of the treatments (three replicates/treatment) of each experiment (performed three times as independent experiments) were statistically analyzed according to Snedecor and Cochran [15] and the mean values of treatments (±SE of three replicates; n = 3) were compared using the least significant difference (L.S.D.) test at the level ($p < 0.05$).

RESULTS AND DISCUSSION

The present work was conducted aiming to achieve an efficient protocol of jojoba regeneration from callus derived from female leaf explants as a prerequisite step for establishing a protocol for *Agrobacterium tumefaciens*-mediated transformation of jojoba plants. Therefore, a set of preliminary experiments were conducted to determine the effect of media composition and the concentrations of various plant growth regulators on callus induction from female leaves of jojoba and the regeneration of shoots from callus. Thus, an improved culture medium for induction of adventitious shoot buds from callus derived from leaves of female jojoba plants was developed and used in this study as shown in (Table 1), in addition to various combinations of plant growth regulators.

Callus Induction from Leaf Explants of Female Jojoba Plants: Sterilized leaf explants about 1 cm^2 were cultured on callus induction medium (CIM) for 8 weeks (with 2 weeks of subculture interval) and then, the produced calli were transferred to bud formation medium (BFM). The obtained results in (Table 2) revealed that, the use of 3, 6-Dichloro-2-methoxybenzoic acid (Dicamba) at (1.5 mg/l) alone led to producing the highest percentage of callus induction (86.06%) which was significantly higher than the obtained percentage (75.67 %) with 2, 4-Dichlorophenoxyacetic acid (2, 4-D) at the same concentration. In addition, the combination of auxin and cytokinin significantly improved the percentage of callus induction and proved to be essential to attain the highest level of callus induction. Moreover the combination between the two auxins 2, 4-D or Dicamba with Thidiazuron (TDZ) was slightly better than that with 6-Benzylaminopurine (BA), while the combination between Dicamba and both cytokinins produced the highest percentage of callus induction (94.50 % with BA and 98.65 % with TDZ) and these percentages were significantly higher than that obtained with 2, 4-D which were (83.14% with BA and 84.27 % with TDZ). Moreover, the produced calli of Dicamba were nodular as shown in (Fig. 1A). Therefore, the Dicamba (1.5 mg/l) was used in combination with TDZ (0.75 mg/l) in the main experiments for shoot regeneration from the produced callus of jojoba leaf explants. The results of the highest percentage of callus induction which were attained (98.65 %) in this study are in agreement with those obtained by Bala *et al.* [12]. Although, the highest percentage of callus induction (98.7%) which was obtained by Bala *et al.,* [12] using 2, 4-D (1 mg/l) + BAP (0.5 mg/l) compared to (98.65 %) which was obtained in this study using Dicamba (1.5 mg/l) + TDZ (0.75 mg/l).

Regeneration of Jojoba Shoots *via* Adventitious Shoot Buds: The produced calli (8-week-old) from leaf explants cultured on CIM supplemented with Dicamba (1.5 mg/l) + TDZ (0.75 mg/l) were transferred to bud formation medium (BFM) supplemented with various combinations of plant growth regulators. It was observed that the calli became more nodular and emerged shoot buds were detected after 2 weeks of culture on (BMF) as shown in (Fig. 1B & C), then after 4 weeks on (BMF) many shoot buds were emerged on callus explants as shown in (Fig. 1D). Afterwards, the emerged shoot buds were proliferated on BFM for more 6 weeks (with 2 weeks of subculture interval) and multiple shoots were developed/callus as shown in (Fig. 1 E-I). Hence, the regenerated shoots produced from one callus were transferred to a jar containing 50 ml of shoot development medium (SDM) supplemented with (TDZ 0.5 mg/l + BA 0.5 mg/l) for 10 weeks (with 2 weeks of subculture interval) as shown in (Fig. 1 J-L). The results in Table 3 showed that, the percentage of shoot regeneration from callus produced from leaf explants of female jojoba plants was significantly increased using the TDZ at the concentration 2.5 mg/l (69.24 %) compared to the same concentration of BA (52.11 %). In addition, the combination between cytokinin and auxin led to a significant increase in the percentage of

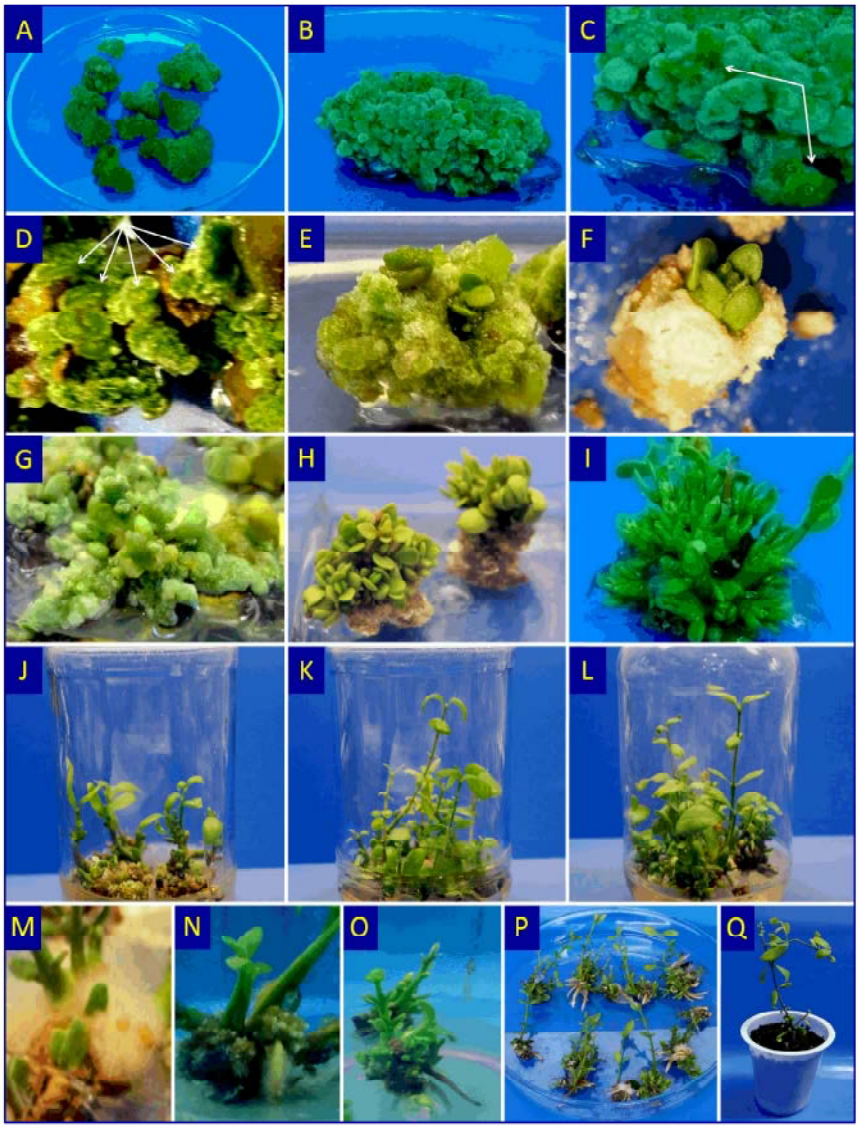

Fig. 1: Regeneration of jojoba female plants *via* adventitious bud formation from callus derived from leaf tissues. (A) Nodular calli after 8 weeks on the callus induction medium. (B) A nodular callus after 2 weeks on bud formation medium (BFM). (C) In focus, arrows point to the developed buds after 2 weeks on (BFM). (D) The proliferated buds after 4 weeks on (BFM). (E) Two emerged shoot buds after 5 weeks on (BFM). (F) An isolated shoot bud after 6 weeks on (BFM). (G) The proliferation of multiple shoot buds after 7 weeks on (BFM). (H) The proliferated shoot buds after 8 weeks on (BFM). (I) The proliferated shoots after 10 weeks on (BFM). (J-L) The regenerated shoots on shoot development medium (SDM), J; after 4 weeks, K; after 8 weeks and L; after 10 weeks. (M) Initiation of root development of regenerated jojoba plants after 2 weeks on root development medium (RDM). (N) The first developed root after 3 weeks on (RDM). (O) Two developed roots after 4 weeks on (RDM). (P) The regenerated jojoba plantlets with developed roots after 6 weeks of culture on (RDM). (Q) An acclimatized regenerated female jojoba plant after 6 weeks in a small pot.

shoot regeneration from callus when compared to the use of cytokinin alone. Moreover, the results of the combination between both types of cytokinin (BA or TDZ) (2.5 mg/l) with Dicamba (0.5 mg/l) which recorded 98.26% and 99.65% respectively, exhibited a slight increase in the percentage of callus produced shoots compared to the use of Naphthylacetic acid (NAA) at (0.5 mg/l) which recorded 97.25% and 98.95% respectively.

Table 2: Effect of auxin (2, 4-D or Dicamba) and cytokinin (BA or TDZ) on callus induction from leaf explants of female jojoba plants

Total No. of explants/ treatment	2, 4-D mg/l	Dicamba mg/l	BA mg/l	TDZ mg/l	% of explants produced callus
297	0.5	-	-	-	55.22±1.91
286	1.0	-	-	-	71.33±1.77
300	1.5	-	-	-	74.33±2.22
294	2.0	-	-	-	73.47±2.11
289	-	0.5	-	-	64.36±1.38
293	-	1.0	-	-	78.84±2.35
287	-	1.5	-	-	84.32±2.67
291	-	2.0	-	-	77.32±2.28
297	1.5	-	0.25	-	79.46±2.41
299	1.5	-	0.50	-	81.61±2.44
294	1.5	-	0.75	-	83.33±2.51
290	1.5	-	1.00	-	82.41±2.46
283	1.5	-	-	0.25	79.86±2.47
295	1.5	-	-	0.50	81.36±2.37
288	1.5	-	-	0.75	84.38±2.56
296	1.5	-	-	1.00	83.11±2.49
289	-	1.5	0.25	-	87.54±2.68
286	-	1.5	0.50	-	91.61±2.77
291	-	1.5	0.75	-	94.50±2.89
298	-	1.5	1.00	-	90.94±2.78
285	-	1.5	-	0.25	88.42±2.73
291	-	1.5	-	0.50	95.19±2.85
296	-	1.5	-	0.75	98.65±2.96
288	-	1.5	-	1.00	95.83±2.79
L.S.D. at 0.05					9.61

Total No. of explants used in three independent experiments which about (100 explants/experiment; divided into three replicates). The data in the columns (Mean ± SE of three replicates; $n = 3$)

While the average number of the produced shoots/callus recorded a significant increase (23.18 shoots/callus) with (TDZ 2.5 mg/l + Dicamba 0.5 mg/l) compared to (TDZ 2.5 mg/l + NAA 0.5 mg/l) which recorded (17.95 shoots/callus), as well as, (BA 2.5 mg/l + Dicamba 0.5 mg/l) recorded (18.15 shoots/callus) compared to (BA 2.5 mg/l + NAA 0.5 mg/l) which recorded (14.06 shoots/callus). Overall, the use of TDZ in combination with Dicamba produced the highest level of shoot regeneration (99.65%) with the highest average number of the produced shoots/callus (23.18).

The obtained results in this study proved that an efficient regeneration protocol of jojoba *via* adventitious bud induction was developed and these results are in accordance with those obtained by Bala *et al.* [12]. Although, the highest number of shoots (14.44) was obtained using (BAP 2 mg/l + NAA 0.2 mg/l) as reported by Bala *et al.* [12] compared to the highest average number of the produced shoots/callus (23.18) which was obtained in this study using (TDZ 2.5 mg/l + Dicamba 0.5 mg/l).

Root Regeneration and Acclimatization of Regenerated Jojoba Plants: The regenerated shoots of jojoba were transferred to root development medium (RDM) and the initiation of root development of regenerated jojoba plants was observed after 2 weeks, then the development of the regenerated roots was completed in 6 weeks (with 3 weeks of subculture interval) as shown in (Fig. 1 M-P). Afterwards, the regenerated jojoba plantlets with strong roots were transferred to small pots filled with a mixture of peat moss and fine sand (3:1) and kept for acclimatization in a growth chamber as described in materials and methods. The successful of rooting stage by developing the roots for the regenerated jojoba shoots is the limiting factor for the success of the regeneration protocol. Thus, various experiments using individual auxin (IBA or NAA) or combination of both (IBA + NAA) or combination of both (IBA + NAA) in addition to cytokinin (BA or TDZ) were conducted as shown in (Table 4).

Hence, the use of individual auxin Indole-3-butyric acid (IBA) at 2.5 mg/l resulted in the highest percentage of shoots produced roots (34.15 %) compared to (NAA) at 3 mg/l which produced (28.28 %), while the combination of both (IBA + NAA) led to increasing the percentage of shoots produced roots up to (58.13 %) as shown in (Table 4). Interestingly, the inclusion of cytokinin (BA 1 mg/l) in combination with (IBA 1.5 mg/l + NAA 1.5 mg/l) led to a significant increase in the percentage of shoots produced roots and recorded the highest level (100.00 %)

Table 3: Effect of cytokinin (BA or TDZ) and auxin (Dicamba or NAA) on shoot regeneration from callus produced from leaf explants of female jojoba plants

Total No. of calli/ treatment	BA mg/l	TDZ mg/l	Dicamba mg/l	NAA mg/l	% of callus produced shoots	Average No. of shoots/callus
283	1.5	-	-	-	34.25±0.74	4.15±0.07
291	2.0	-	-	-	43.47±1.29	5.98±0.12
287	2.5	-	-	-	52.11±1.55	7.22±0.16
296	3.0	-	-	-	46.22±1.33	4.62±0.11
281	3.5	-	-	-	41.58±1.21	3.18±0.06
298	-	1.5	-	-	35.14±0.81	7.84±0.18
293	-	2.0	-	-	46.29±1.31	9.58±0.23
285	-	2.5	-	-	69.12±1.86	11.78±0.31
280	-	3.0	-	-	50.45±1.41	8.64±0.22
291	-	3.5	-	-	42.65±1.26	7.29±0.19
292	2.5	-	0.25	-	87.67±2.15	12.69±0.34
287	2.5	-	0.50	-	98.26±2.61	18.15±0.47
295	2.5	-	0.75	-	91.19±2.32	14.25±0.36
289	2.5	-	1.00	-	85.12±2.19	11.43±0.30
283	2.5	-	-	0.25	84.45±1.17	10.37±0.27
291	2.5	-	-	0.50	97.25±2.48	14.06±0.34
286	2.5	-	-	0.75	87.41±2.23	12.54±0.29
294	2.5	-	-	1.00	81.63±2.08	11.21±0.28
292	-	2.5	0.25	-	92.46±2.37	15.96±0.42
287	-	2.5	0.50	-	99.65±2.86	23.18±0.57
297	-	2.5	0.75	-	96.30±2.56	18.72±0.49
289	-	2.5	1.00	-	94.46±2.52	16.84±0.44
297	-	2.5	-	0.25	91.92±2.34	12.69±0.32
285	-	2.5	-	0.50	98.95±2.62	17.95±0.46
288	-	2.5	-	0.75	92.36±2.39	15.22±0.41
295	-	2.5	-	1.00	87.46±2.35	13.87±0.36
L.S.D. at 0.05					12.35	3.73

Total No. of calli used in three independent experiments which about (100 callus/experiment; divided into three replicates). The data in the columns (Mean ± SE of three replicates; $n = 3$).

compared to the combination of (IBA 1.5 mg/l + NAA 1.5 mg/l) which recorded (58.13 %). In addition, the comparison between the effect of the type of cytokinin on root formation elucidated that, the use of BA in combination with (IBA+NAA) was more efficient than the combination between TDZ and (IBA+NAA) which resulted in the highest percentage of shoots produced roots (100.00 %) and (94.21 %), respectively. Also the inclusion of BA or TDZ in combination with (IBA+NAA) resulted in significant increases in the average number of regenerated roots/plantlet which recorded the highest level (7.11 roots/plantlet) with the combination between BA and (IBA+NAA) followed by the (6.22 roots/plantlet) with the combination between TDZ and (IBA+NAA) as shown in (Table 4).

Moreover, in total 1784 plants were obtained after 10 weeks of successful acclimatization stage which achieved on two steps; the first step was carried out in the growth chamber for 6 weeks followed by the second step which was performed in the greenhouse for 4 weeks. Thus, the average number of regenerated roots/plantlet was positively correlated with the percentage of the survival rate of the acclimatized jojoba plants, thus the highest survival rate (96.41 %) was recorded for the plants with the highest average number of regenerated roots/plantlet (7.11) as shown in (Table 4). Generally, significant differences were obtained in many reports when auxin either alone or with other plant growth regulators was used for jojoba root formation [6, 7, 12, 16]. The obtained results of the highest percentage of shoots produced roots which attained (100.00 %) are in concurrence with those obtained by Bala *et al.* [12], although, the highest percentage of shoots produced roots was (92.8 %) with the highest number of roots/plantlet (6.22) using IBA (3 mg/l) compared to the obtained results in this study of the percentage of shoots produced roots (100.00 %) with the highest number of roots/plantlet (7.11) using the combination between BA (1 mg/l) and (IBA 1.5 mg/l + NAA 1.5 mg/l). Finally, The obtained results of the percentage of the survival rate of the acclimatized jojoba plants in this study are in accordance with those obtained by Bala *et al.* [12] with a high percentage obtained here of the survival rate (96.41%) compared to (90 %) which was obtained by Bala *et al.* [12].

Table 4: Effect of auxin (IBA or NAA) and cytokinin (BA or TDZ) on root regeneration of jojoba plants

Total No. of shoots/ treatment	IBA mg/l	NAAmg/l	BA mg/l	TDZ mg/l	% of shoots produced roots	Average No. of roots/plantlet	Survival rate %
244	0.5	-	-	-	03.28±0.07	1.25±0.02	50.00
237	1.0	-	-	-	08.02±0.17	1.21±0.02	47.36
251	1.5	-	-	-	18.33±0.44	1.41±0.03	52.17
242	2.0	-	-	-	29.34±0.69	1.56±0.04	53.52
246	2.5	-	-	-	34.15±0.83	1.76±0.05	54.76
249	3.0	-	-	-	25.70±0.61	1.53±0.04	51.56
240	3.5	-	-	-	19.58±0.47	1.40±0.03	51.06
248	4.0	-	-	-	15.73±0.36	1.33±0.03	51.28
245	-	0.5	-	-	01.63±0.03	1.00±0.00	25.00
247	-	1.0	-	-	04.45±0.09	1.09±0.02	27.27
238	-	1.5	-	-	09.24±0.22	1.23±0.03	27.27
249	-	2.0	-	-	13.25±0.32	1.52±0.04	45.45
257	-	2.5	-	-	21.40±0.51	1.55±0.04	47.27
244	-	3.0	-	-	28.28±0.75	1.66±0.04	53.62
247	-	3.5	-	-	25.91±0.64	1.63±0.03	48.44
243	-	4.0	-	-	22.22±0.53	1.48±0.03	40.74
241	1.0	1.0	-	-	41.49±1.01	3.65±0.08	88.00
246	1.5	1.5	-	-	58.13±1.41	4.38±0.11	89.51
242	2.0	2.0	-	-	47.93±1.27	3.91±0.09	88.79
244	1.0	1.0	1.00	-	65.57±1.74	4.96±0.10	92.50
251	1.5	1.5	1.00	-	100.00±0.00	7.11±0.17	96.41
255	2.0	2.0	1.00	-	81.57±1.82	5.64±0.12	94.23
254	1.0	1.0	-	1.00	60.63±1.48	4.57±0.11	90.26
259	1.5	1.5	-	1.00	94.21±2.42	6.22±0.13	95.08
242	2.0	2.0	-	1.00	76.03±1.61	4.95±0.12	91.85
L.S.D. at 0.05					24.08	1.58	16.77

Total No. of shoots used in three independent experiments which about (80 shoots/experiment; divided into three replicates). The data in the columns (Mean ± SE of three replicates; $n = 3$)

Here it is worthy to mention that, the use of the nutrient solution (1/4 strength of MS medium) for irrigation of jojoba plants and the daily foliar application of plants with (1/10 strength of MS) supplemented with (BA 1 mg/l + TDZ 1 mg/l + GA3 0.5 mg/l) during the acclimatization period proved to be essential to obtain the highest percentage of the survival rate which recorded in this study. Hardening of regenerated jojoba plants before transfer to the field increases the percentage of the survival rate of the transferred jojoba plants [6-8, 17].

The obtained results in this study proved that a highly efficient regeneration protocol of jojoba plants *via* adventitious shoot bud formation from callus derived from female leaf explants was achieved. As well as the high capacity of jojoba shoot regeneration which attained (99.65 %) may be attributed to the combinations of the plant growth regulators and the composition of the improved culture medium which containing low levels of ammonium (in CIM; 14.63 mM, in BFM and SDM; 2 mM and in RDM; 7.31 mM) compared to the MS medium according to Murashige and Skoog [13] which contains (20.63 mM), in this context, Hamama *et al.* [9] used a 1/2 strength of MS (ammonium; 10.32 mM) for somatic embryogenesis in callus derived from jojoba leaf explants, while Gaber *et al.* Mohammed *et al.* and Bala *et al.* [10-12] used a full strength of MS (ammonium; 20.63 mM). In addition, this improved medium contains the reduced levels of chloride (in CIM, BFM and SDM; 5.99 µM and in RDM; 2.99 µM) in comparison to 5.99 mM in MS medium due to the presence of calcium chloride ($CaCl_2.2H_2O$) 444 mg/l as shown in Table 1, whereas the sensitivity of plant species and in particular woody plant species to Cl^- toxicity was reported widely [18]. Also, the effect of chloride toxicity on *in vitro* culture of plants was previously reported; for woody plants according to McCown and Sellmer [19], *Prunus avium* according to Pevalek-Kozlina and Jelaska [20], *Ocimum basilicum* [21]. While the level of copper was elevated to 5 µM compared to 0.1 µM in MS medium, in addition to silver nitrate (5.9 µM), whereas, these anti-ethylene substances in addition to the $CoCl_2$ in MS medium play an important role in down-regulating ethylene biosynthesis, thus, these

substances proved to be essential for regeneration of recalcitrant plants [21-30]. Furthermore, the improved medium was supplemented with spermidine (1 mg/l) which involved in signal transduction pathways during embryogenesis in plants [31]. Also, casein hydrolysate (300 mg/l), as a source of all amino acids for protein biosynthesis, myo-inositol (250 mg/l), B5 vitamins according to Gamborg *et al.* [14] and additional vitamins were added to this improved medium, in addition to adenine sulphate 50 mg/l in (BFM and SDM).

CONCLUSIONS

Overall, the obtained results in this study proved that a highly efficient regeneration protocol of female jojoba plants *via* adventitious shoot bud formation from callus derived from leaf explants with a high regeneration capacity of jojoba plants (99.65 %) was achieved. The success of this protocol in producing regenerated jojoba plants at the high rate may be attributed to several factors; (a) the young leaves of female plants which were used as explants, (b) the improved medium including all supplements and the sterilization of all culture media through sterilized 0.22 µm filters, in addition to (c) the obtained combinations of plant growth regulators examined in this work. Moreover, this improved protocol of jojoba regeneration from leaf explants *via* adventitious shoot bud formation is of great importance for establishing a reliable protocol for *Agrobacterium tumefaciens*-mediated transformation of jojoba plants.

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