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Effect of Growth Regulators and Salinity Levels on *In vitro* Cultures of Jojoba Plants

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Abstract: The present investigation aimed to induce shoot multiplication and callus cultures of jojoba (*Simmondsia chinensis* Link (Schneider)) distinguished clone. To investigate the induction of callus culture, *in vitro* leaves of jojoba clone, with two different sizes, were cultured on MS medium supplemented with NAA alone or combined with BAP. Various growth regulators (NAA, IBA+BAP, 2, 4 D+BAP or NAA+BAP) were used to proliferate callus cultures. Clusters of jojoba clone derived from *in vitro* cultures were also cultured on MS medium supplemented with various concentrations of BAP to enhance shoot multiplication. In addition, low levels of diluted seawater (0, 500, 1000, 1500 and 2000 ppm) were investigated on callus and shoot multiplication. Results indicated that NAA with BAP induced callogenesis, while NAA alone enhanced proliferation. Furthermore, multiplication of *in vitro* jojoba shoots could be highly achieved by 4.0 mg/L BAP. Increasing seawater levels in proliferation medium increased callus fresh weight as well as shoot multiplication. Oil content of callus cultures was affected by growth regulators and seawater levels. In addition, leaf chlorophyll a, b and carotinoids, total indoles, phenols, sugars and proline content of resulted plantlets could explain the results obtained.

Key words: Biofuel plant · Callogenesis · Growth regulators · Jojoba · Micropropagation · Salinity

INTRODUCTION

Salinity is a major problem especially in arid and semi arid zones. About seven percent of the world's total land area is affected by salinity [1]. Most importantly, the percentage of cultivated land affected by salt is even greater. Furthermore, there is a dangerous trend of 10 % per year increase in the saline area throughout the world [2]. Egypt is one of the countries that suffer severe salinity problems. For example, 33% of the cultivated land [3], which comprises only 3% of total land area in Egypt, is already salinized [4]. The reduction in production of soils affected by salinity is about 30% [5]. Therefore, cultivation of salt-tolerant crops has become an urgent task in dealing with salinity problems. Moreover, selection of new economic crops which can grow in salinized area is an important goal. Jojoba (*Simmondsia chinensis* (Link) Schneider) or hohoba belongs to family Simmondsiaceae and tolerates salinity. It is an evergreen, dioeciously desert shrub, native of Sonoran desert, north-west Mexico and Baja California [6]. The seeds of jojoba which are used as a drink like coffee in its natural nation contain about 50% oil by weight. Jojoba oil resembles sperm whale oil and is resistant to degradation by bacteria. It is a natural high temperature and high-pressure lubricant [7]. It is edible and contains simmondisin, which depresses appetite. It does not grow rancid and may become suitable for vegetable oil. Jojoba is propagated by sexual and vegetative methods. In plant populations derived by sexual propagation, it is difficult to determine sex type in early stages of growth, due to their dioccious nature. These plants are genetically variable, which affects growth uniformity, yield and early bearing [8] as well as oil yield. In addition, a 5: 1 female to male ratio needed at the field, as reported earlier [9] is yet another major problem that obstructed large-scale propagation of desired plants of jojoba. The vegetative propagation of jojoba via conventional stem cuttings did not prove effective due to

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long procedure and slow growth [6, 10]. Due to its dioccious 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 commercial cultivation [11]. However, jojoba oil was not only obtained from seeds but also from various explants and *in vitro* callus cultures [12]. Oil from callus, if extracted by suitable methods [13], can minimize our dependence on seeds for oil production [14, 15]. So that, proliferation of callus cultures would be another need.

With the above background in view and increasing commercial demand of jojoba oil in Egypt, the present investigation aims to improve micropropagation of jojoba explants and callus cultures. In addition, the effect of low levels of seawater was investigated.

MATERIALS AND METHODS

The present study was carried out throughout 2013-2014 at Biotechnology of Fruit Trees Laboratory, Pomology Department, National Research Centre, Dokki, Giza, Egypt to investigate the following issues:

Callus Cultures:

Callus Induction: It is aimed to investigate the effect of growth regulators, explant size and number of subcultures on callogenesis from jojoba leaves of a distinguished clone. Jojoba *in vitro* leaves with two size (0.5 or 1.0 cm in width) were cultured into MS medium [16] supplemented with naphthalene acetic acid (NAA) at 1.0 mg/L alone or combined with benzyl aminopurine (BAP) at 1.0 mg/L. Percentage of responded explants (explants show callus induction) was determined. In addition, the amount of callus induced was determined visually according to Pottino [17] as, 1= no callus, 2= low, 3= moderate, 4= high and 5= very high.

Callus Proliferation:

Growth Regulator: It is aimed to investigate the effect of NAA, NAA with BAP, indole butyric acid (IBA) with BAP or 2,4-Dichloro-phenoxy acetic acid (2,4 D) with BAP all at 1.0 mg/L on proliferation of callus cultures. Induced callus (1.0 g) was used for each replicate and the growth responses were determined based on fresh weight of callus cultures at subculture two and subculture eight.

Seawater Levels: It is aimed to investigate the effect of low levels of seawater (41000 mg\L) at 0, 500, 1000, 1500 and 2000 ppm on jojoba callus cultures. MS medium was supplemented with NAA at 1.0 mg/L. One gram of jojoba callus was used for each replicate. Growth responses were determined based on fresh weight of callus cultures after two and four subcultures.

Oil Extraction: Oil content was extracted from callus produced from previous experiments after four subcultures to study the effect of type of growth regulator as well as seawater levels according to the method of A.O.C.S. [18].

Multiplication of Jojoba Shoots:

BAP Concentration: Shoots of the distinguished jojoba clone were cultured on ¾ MS medium [11] supplemented with BAP at 0.0, 2.0 and 4.0 mg/L. Three clusters, each containing two shoots were cultured in each jar (200 mL) which representing one replicate. The average shoot number, shoot length and leaves number per shoot were recorded.

Seawater Levels: It is aimed to investigate the effect of seawater levels on multiplication of jojoba plants. Shoot tips of jojoba clone derived from *in vitro* cultures were cultured on MS medium supplemented with 4.0 mg/L BAP. Low levels of diluted seawater (0, 500, 1000, 1500 and 2000 ppm) were investigated. The growth response was determined based on average shoot number, shoot length and leaves number.

Physiological Analysis: *In vitro* shoots presented to seawater levels were collected and prepared for analysis. Chlorophyll a, b and carotenoids, total indoles and phenols were determined according to the method of Arnon [19]. Total soluble sugars (TSS) were determined according to the method of Fales [20]. Shoot proline content was determined by a spectrophotometric assay as described by Bates *et al*. [21].

Rooting Stage: *In vitro* shoots from previous treatments were cultured on ¾ MS medium supplemented with NAA at 1.0 mg/L + IBA at 7.0 mg/L [11] to induce rooting. Rooted plantlets were acclimatized in greenhouse.

Statistical Analysis: Complete randomized design was adopted with 3 replicates for each treatment. Data were statistically analyzed according to Duncan's multiple range test at 5% level of probability [22].

RESULTS

Callus Culture:

Callogenesis: Data in Table 1 and Fig.1 revealed that explants size affected the percentage of responded explants to show callogenesis and callus degree. Explants with big size (1.0 cm, in width) gave a percent higher than that with small size (0.5 cm, in width), approximately the double. Considering growth regulators, the presence of BAP with NAA increased the percentage of responded explants as well as callus degree. With respect to the interaction, the highest percentage of responded explants was from the big explants size with growth regulators combination while, the lowest percentage appeared from the small size with NAA only. Meanwhile, there is no difference between the callus degree of the big and the small explant size within the same growth regulator treatment. The combination between NAA and BAP gave higher degrees than NAA alone. Data in Table 2 indicated that, number of subcultures affected the percentage of responded explants and callus degree. It is obvious that subculture four gave a higher percentage as well as callus degree than subculture two. In addition, the presence of BAP with NAA increased the percentage of responded explants and callus degree. With respect to the interaction, the highest percentage of explants show response was appeared with subculture four and growth regulators combination while, the lowest percentage appeared with subculture two and NAA only. Considering callus degree, there were no significant differences between the interactions.

Callus Proliferation

Growth Regulators Combinations: Data in Table 3 Fig. 1 revealed that, transferring callus cultures for eight subcultures multiplied the fresh weight four folds, approximately, compared with that of subculture two. Furthermore, presenting callus cultures to NAA alone gave the highest fresh weight followed by NAA+BAP then other treatments. The interactions of subculture eight with various growth regulators showed surpassed results compared with those of subculture two. The highest callus fresh weight was achieved with using NAA alone at subculture eight while, the lowest callus fresh weight was achieved with using IBA with BAP at subculture two.

Seawater Levels: Data in Table 4 and Fig. 1 indicated that, presenting callus cultures to seawater levels surpassed fresh weight as; the level of 2000 ppm gave the highest fresh weight. Meanwhile, the lowest callus fresh weight was appeared with the control. Callus fresh weight achieved from subculture four show higher values compared with that of subculture two, approximately the double. Concerning the interaction between number of subcultures and levels of seawater, results show that, the level of 2000 ppm gave the highest fresh weight at subculture four followed by the level of 1500 ppm at the same subculture. It could be observed that, the level of 2000 ppm surpassed the fresh weight at subculture two compared with that of the control at subculture four make it appear that it fastener the callus growth to surpass the result of subculture four.

Table 1: Effect of explant size and growth regulators on inducing callogenesis.

Parameters	Responded explants %			Callus degree		
GR treatments	Big size (1.0 cm)	Small size (0.5 cm)	Mean	Big size (1.0 cm)	Small size (0.5 cm)	Mean
NAA	41.00	20.90	30.95	1.35b	1.38b	1.37B
NAA+BAP	62.50	27.00	44.75	2.33a	2.00a	2.17A
Mean	51.75	23.95	--	1.84A	1.69B	--

Means with different letters within each parameter were significantly different at 5% level, GR: growth regulators.

Table 2: Effect of growth regulators and subculture number on inducing callogenesis.

Parameters	Responded explants %			Callus degree		
GR treatments	Subculture two	Subculture four	Mean	Subculture two	Subculture four	Mean
NAA	35.8	48.28	42.04	1.36a	1.89a	1.63B
NAA+BAP	36.0	82.81	59.41	1.83a	2.15a	1.99A
Mean	35.9	65.55	--	1.60B	2.02A	--

Means with different letters within each parameter were significantly different at 5% level, GR: growth regulators.

Table 3: Effect of growth regulators combinations and subculture on callus culture fresh weight (g).

Growth regulators	Subculture two	Subculture eight	Mean
2,4-D+BAP	5.89c	14.96b	10.43C
NAA+BAP	6.04c	16.63b	11.34B
NAA	4.04cd	18.80a	11.42A
IBA+BAP	3.43d	14.98b	9.21D
Mean	4.85B	16.34A	

Means with different letters were significantly different at 5% level.

Table 4: Effect of low levels of seawater and subculture on callus culture fresh weight (g).

Seawater levels	Subculture two	Subculture four	Mean
Control	2.55f	3.32def	2.94E
500 ppm.	2.77f	4.01def	3.39D
1000 ppm.	2.98ef	4.72cde	3.85C
1500 ppm.	5.08cd	12.17b	8.63B
2000 ppm.	6.03c	15.37a	10.7A
Mean	3.88B	7.92A	--

Means with different letters were significantly different at 5% level.

Table 5: Effect of BAP concentration on multiplication of *in vitro* jojoba shoots after four subcultures.

BAP conc.	Shoot number	Shoot length (cm)	Leaves number
0.0	4.50c	1.64a	4.78c
2.0	9.11b	1.10b	6.10b
4.0	12.84a	0.83c	6.49a

Means with different letters within each parameter were significantly different at 5% level.

Table 6: Effect of low levels of seawater on multiplication of *in vitro* jojoba shoots after four subcultures.

Seawater levels	Shoot number	Shoot length (cm)	Leaves number
Control	11.50d	0.76d	3.50a
500 ppm	11.50d	1.38a	3.28b
1000 ppm	12.50c	1.28b	3.10c
1500 ppm	13.75b	1.12c	2.92d
2000 ppm	16.50a	1.10c	2.78e

Means with different letters within each parameter were significantly different at 5% level.

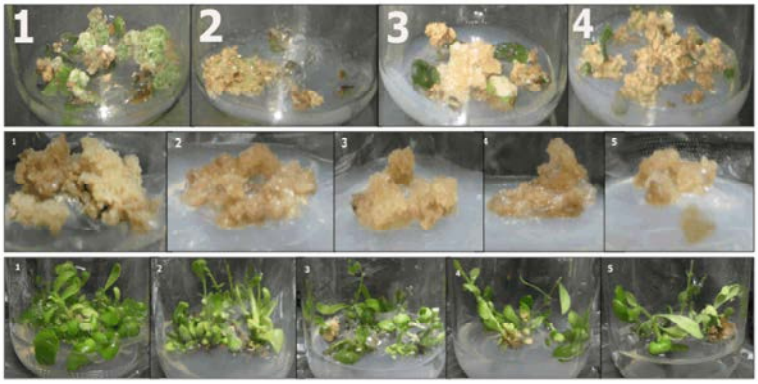

Fig 1: First row: callus induction (1) explants with big size+ NAA, (2) small size+ NAA, (3) big size+ NAA+ BAP, (4) small size+ NAA+BAP. Second and third row are callus cultures and shoot multiplication, respectively. Treatments 1,2,3,4 and 5 were seawater levels: 2000, 1500, 1000, 500, and 0.0 ppm, respectively.

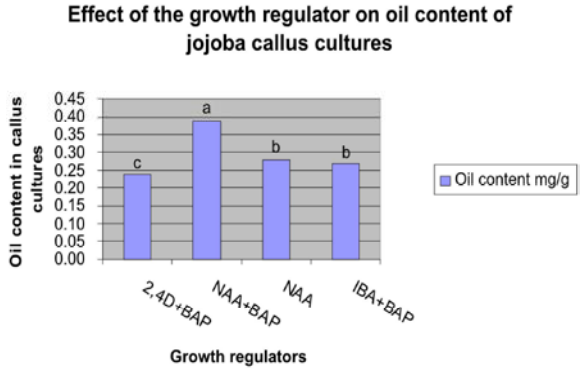

Fig. 2: Effect of growth regulator type on oil content of jojoba callus tissues.

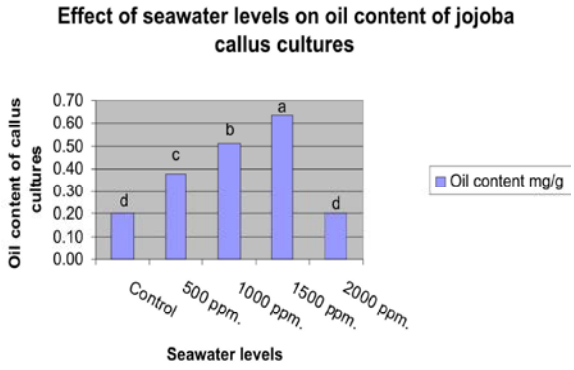

Fig. 3: Effect of low levels of seawater on oil content of jojoba callus tissues.

Table 7: Effect of low levels of seawater on physiological analysis of *in vitro* jojoba shoots after four subcultures.

Seawater levels	TSS %	Total indoles (mg/g FW)	Total phenols (mg/g FW)	Chl. *a* (mg/g FW)	Chl. *b* (mg/g FW)	Carotenoids (mg/g FW)	Proline (mg/g DW)
Control	22.3b	0.025d	0.611a	0.30b	0.10d	0.062a	3.087e
500 ppm	21.7c	0.375c	0.455b	0.20c	0.10d	0.071a	4.223a
1000 ppm	21.6c	0.425b	0.535b	0.19c	0.40c	0.043a	4.090b
1500 ppm	22.4b	0.425b	0.495b	0.30b	0.56b	0.065a	3.980c
2000 ppm	29.9a	0.450a	0.603a	0.60a	0.80a	0.103a	3.250d

Means with different letters within each column were significantly different at 5% level, FW: fresh weight, DW: dry weight.

Oil Content

Growth Regulators Combinations: Data presented in Fig. 2 indicated that growth regulator type and combination affected oil content values of jojoba callus tissues. It could be observed that the combination between NAA and BAP enhanced oil content, significantly, followed by NAA and IBA+BAP while, the combination of 2, 4-D+BAP caused the lowest oil content.

Seawater Levels: Data presented in Fig. 3 showed that seawater levels affected oil content values of jojoba callus cultures. The highest oil content was detected at 1500 followed by 1000 than 500 ppm, significantly. Meanwhile, the lowest oil content was detected at the control or the highest level of seawater.

Shoot Multiplication:

Effect of BAP Concentration: It is clear from Table 5 that, higher concentration of BAP (4.0 mg/l) resulted in significant increase in shoot and leaves numbers as compared with the lower level and the control. Meanwhile, presenting shoots to the tested BAP concentrations showed lower shoots length compared with the control.

Effect of Low Levels of Seawater: It is obvious from Data in Table 6 that, presenting *in vitro* jojoba shoots to the highest level of seawater (2000 ppm) showed the highest shoot number compared with either levels used or the control. However, the level of 500 ppm showed the highest shoot length followed by other levels while, the lowest length was appeared with the control. Meanwhile, with increasing seawater levels leaf number gradually decreased showing the lowest number with the highest level used.

Physiological Analysis: Data in Table 7 indicated that, TSS% increased with the highest level of seawater (2000 ppm) while lower levels gave the same or lower TSS compared with the control. Similarly, the level of 2000 ppm gave the highest total indoles, phenols, chlorophyll *a* and *b* contents compared with other levels of seawater. However, caroteniods content did not show any differences to either seawater levels. In the other hand, all levels of seawater gave higher proline content compared with the control but, it was decreased gradually by increasing levels of seawater over 500 ppm.

DISCUSSION

The previous data indicated that, explants with big size and multiple subcultures showed increasing in induction of callus from jojoba *in vitro* leaves. In addition, combination between NAA and BAP both at 1.0 mg/L enhanced callus induction. These results are similar to the findings of Shahsavari *et al.* [23], who stated that, rice callus growth became more evident with the subculture two than with the first subculture. Singh *et al*. [24] revealed that, earliest and maximum (99%) callus induction response was observed from *Catharanthus roseus* explants on MS medium supplemented with BAP and NAA both at 1.0 mg/L. It is concluded that auxins and cytokinins both play an important role in regulating the induction of jojoba callus [12]. However, in proliferation stage, NAA alone with multiple subcultures show the highest fresh weight of multiplied callus cultures. Furthermore, increasing levels of seawater gradually increased average fresh weight of proliferated callus. Similarly, presenting callus cultures of *Cymbopogon martini* to 10% v/v seawater in the medium increased callus yield (fresh weight) [25]. In addition, increased dry weight of the callus cultures of Miswak (*Salvadora persica* Linn.), soluble proteins, proline, soluble carbohydrates and catalase activity were recorded under NaCl stress in comparison to control cultures [26].

The present data indicated that growth regulator type and combination as well as seawater levels affected oil content values of jojoba callus tissues. Similarly, among factors influencing essential oil production are plant growth regulators or plant hormones. Endogenous levels as well as exogenous application could affect essential oil production and chemical composition [27]. Increasing salinity gradually enhanced the oil contents in shoots and

flowers of *Chamomilla recutita* and in roots and shoots of *Origanum majorana* [28]. The present investigation indicated that, multiplication of *in vitro* jojoba shoots could be achieved by 4.0 mg/L BA. In addition, presenting *in vitro* jojoba shoots to seawater levels increased shoots multiplication significantly. These findings were similar to those obtained by Roussos *et al.* [29] when compared different concentrations of BAP (1.0, 2.0, 4.0, 6.0 and 8.0 mg/L). They found that 4.0 mg/L concentration enhanced shoot proliferation in jojoba shoot tip and node culture. Meanwhile, Tyagi and Prakash [30] reported that all tested concentrations of BA (0.5, 1.0 and 2.0 mg/L) enhanced shoot proliferation from nodal explants of five jojoba genotypes male and female plants and 2.0 mg/L BAP proved to be optimum for shoot regeneration. Regarding to salinity effect, the *in vitro* shoot length, fresh weight and root length of MM.106 apple rootstock was increased at 20 mM NaCl, significantly, as compared with the concentrations of 40, 80, 100, 120 mM and the control [31]. Moreover, with 4 grapevine cultivars, Charbaji and Ayyoubi [32] found that shoot length and leaf number of three cultivars were significantly increased at low salt levels, whereas high salt concentrations decreased all growth indicators and organic compounds at all cultivars.

The present data showed that, the highest level of seawater (2000 ppm) gave the highest total TSS %, total indoles, phenols, chlorophyll *a* and *b* content compared with other levels of seawater. These results are in harmony with the finding of Hamed and Ali [33]. They found that salinity treatment stimulated accumulation of total soluble sugars compared with control. Addition of KCl to the compact callus cluster cultures established from *Catharanthus roseus* markedly improved indole alkaloid production [34]. However, Ahmad *et al*. [35] stated that NaCl stress was studied in leaves of two varieties of pea (*Pisum sativum* L.) cv. EC 33866 and Puget. High chlorophyll stability was observed in EC 33866 varieties than Puget. In *Pisum arvense,* the phenolic content increased slightly compared to control till it reached 120 mM NaCl. At higher concentrations the phenolic content decreased while, in *P. sativum,* the phenolic content at 75 mM NaCl was at its maximum and further decreased. In *P. fulvum,* the phenolic contents in control and at 75 mM NaCl were almost the same and they decreased at higher rates of NaCl [36]. Increasing proline content compared with the control due to seawater levels is in harmony with the findings of Saeed *et al.* [37], who studied the effect of different salt concentrations (5000, 7000, 10000 and 12000 mg/L) on jojoba seedlings. Proline and total sugar contents increased significantly as salt concentrations increased. In addition, sharp increase in the accumulation of proline was observed in two varieties of *Pisum sativum* L. at all NaCl stress regimes [35]. Apart from protection of macromolecules from denaturizing and carbon and nitrogen reserve for stress relief, proline has several other functions during stress, e.g. osmotic adjustment [38], osmo-protection [39], free radical scavenger and antioxidant activity [40].

CONCLUSION

From the previous results it can be concluded that salinity tolerated plants, especially jojoba, could be growth enhanced by presenting them to low level of salinity. Physiological analysis proved that low levels of salinity did not inhibit growth and multiplication of callus and shoots of jojoba *in vitro* cultures. Jojoba liquid wax could be achieved from jojoba callus cultures and need further treatments for production enhancement.

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