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In vitro Conservation of Jojoba (*Simmondsia chinensis*) Shootlet Cultures Using Osmotic Stress and Low Temperature

Bekheet, S. A., Matter, M.A., H. S. Taha and El-Ashry A. A.

Plant Biotechnology Department, National Research Centre, 33 El Bohouth St., Dokki, Giza PO. 12622, Egypt.

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ABSTRACT

This work was conducted to develop an *in vitro* method for short-term conservation of planting materials of jojoba. Shootlets were *in vitro* proliferated from node explants on MS medium supplemented 1 mg/l BA. The shootlets were stored on medium contained three concentrations of mannitol or polyethylene glycol (PEG). Also, slow growth induced by incubation at low temperature (5 °C) was examined. Pronounced decline in both number of proliferated shootlets and shootlets height were observed on low level of mannitol. However, all PEG treatments and the high levels (40 and 60 g/l) of mannitol caused a progressive reduction of the two growth parameters. It was noticed that, the growth parameters were more decreasing in the presence of PEG comparing to that registered in the presence of mannitol. The lowest values of shootlets number (1.50) were registered with 15 g/l PEG containing medium. However, slight increasing of branches was recorded with mannitol containing medium. Also, low temperature has tended to reduce both numbers of shootlets and shootlets height. However, the regenerated branches of jojoba shootlets were obviously enhanced by low temperature. On the other hand, the high level of mannitol (60 g/l) and the three levels (5, 10 and 15 g/l) of PEG caused shootlet necrosis. On contrast no necrosis was observed with the three periods of low temperature storage.

Key words: Jojoba shootlets, *in vitro* storage, mannitol**,** polyethylene glycol, low temperature.

Introduction

Jojoba (*Simmondsia chinensis*) plant is an arid perennial shrub that adapted to hot and dry climates (Hogan, 1978). Jojoba seed is rich in liquid wax, commonly mistaken for jojoba oil (Van Boven *et al*., 1997). The physical properties of jojoba oil are: high viscosity, high flash and fire point, high dielectric constant, high stability and low volatility. The cosmetic industry appears to be the principal market for jojoba oil products, and the other major industry using jojoba oil is the pharmaceutical sector. Jojoba is dioecious; the female plants produce seed from flowers pollinated by the male plants. Female plants are commercially more important for the seed production (Harsh *et al.*, 1987). Vegetative propagation enables the establishment of plantations with the desired proportion of male to female plants of pre-selected superior clones. It has four main advantages over seed propagation of jojoba; (a) higher and more uniform yield, (b) early bearing of fruit, (c) reduced costs of later cultural and harvesting operations and (d) evolution of true to type uniform, desirable clonal variety for future multiplication (Hogan and Palzkill, 1983). Rooting of stem cuttings is the most commonly used asexual propagation method in jojoba (Palzkill and Feldman, 1993). The best cutting material is the current year growth that is semi-hardened (Palzkill, 1988; Prat *et al*., 1998; Bashir *et al*., 2001). Despite success in conventional vegetative propagation, the amount of shoot material for cuttings is limited and rooting is hard and lengthy process. Otherwise, clonally propagated jojoba plants are usually maintained in the field. Such a procedure is laborious and exposes plants to pests and environmental stresses.

Micropropagation of jojoba is a very promising means of mass production of superior clones for commercial plantations. In this respect, several reports on *in vitro* propagation appeared in literature using various explants such as axillary buds (Chaturved and Sharma, 1989; Llorente and Apostolo, 1998) and shoot tip (Sardana and Batra, 1998). Otherwise, the differential morphogenic response of nodal explants of male and female jojoba clones on media supplemented with different cytokinins has been reported by Agrawal *et al*., (1999). Moreover, maturation and germination of somatic embryos from leaf tissue of jojoba was recognized (Hamama *et al*., 2001). Otherwise, *in vitro* slow growth culture is used as an

Corresponding Author: El-Ashry A. A., Plant Biotechnology Department, National Research Centre, 33 El Bohouth St., Dokki, Giza PO. 12622, Egypt.
E-mail: aaelashry@gmail.com

alternative method for short- to medium-term storage that can eliminate the obstacles of field conservation. Using the *in vitro* preservation with jojoba is very valuable, as reproduction by seed does not yield true-to-type plants. In this respect, minimal growth conditions for short to mid-term storage are achieved by induction of osmotic stress with sucrose or mannitol (Westcott, 1981), reduced temperature and/or light (Withers, 1991) and incorporation of sub-lethal levels of growth retardant (Mandal, 1995; Gupta, 2001). In general, the most widely applied technique for slow growth preservation is temperature reduction, which can be combined with a reduction in the concentration of nutritive elements and a decrease in light intensity or storage in the dark (Engelmann, 1997). In this context, several types of plant materials have been used for *in vitro* preservation of clonally propagated crops. Storage of stem nodes explants under slow rate of growth has a significant use in the international germplasm resources units. This procedure makes germplasm available at any times for international distribution (Wilkins and Dodds, 1983).

The aim of this work was to evaluate the influence of addition of mannitol or polyethylene glycol to culture medium and low temperature incubation condition as slow growth factors on storage of jojoba shootlets grown *in vitro.*

Materials and Methods

Establishment of in vitro shootlet cultures

Vegetatively propagated jojoba plants were used as plant materials. Nodes were cut from branches and washed by running tap water for 2 hours and then sterilized with ethanol 70% for 30 second and Clorox (50 %) for 10 minutes. The nodes were washed with sterile water under laminar air-flow hood. Explants of 0.5 cm long were excised and cultured into 350 ml glass jars containing (MS) medium Murashige and Skoog (1962) supplemented with 1 mg/l benzyl adenine (BA) for five weeks. The proliferated shootlets were subcultured on fresh medium contained same compositions two times (five weeks interval) for shootlets multiplication (Bekheet *et al*., 2015) which they were used for preservation experiments.

Effect of mannitol on shootlets preservation

To evaluate the effect of mannitol on *in vitro* preservation of jojoba shootlets, uniform *in vitro* grown shootlets (1cm length) were transferred to multiplication medium (MS + 1 mg/l BA) supplemented with 20, 40 and 60 mg/l of mannitol. The cultures were incubated at normal growth conditions. Growth parameters i.e., number of proliferated shootlets, shootlets high (cm), numbers of branches and necrosis percentages were registered after 9 months of storage.

Effect of polyethylene glycol on shootlets preservation

To examine the effect of polyethylene glycol (PEG) on slow growth storage of jojoba, the *in vitro* multiply shootlets were taken in 1 cm length and cultured on MS medium containing 1 mg/l BA and amended with 5, 10 and 15 g/l of PEG (400). The growth parameters were recorded as in the previous experiment after 9 months of preservation at normal growth conditions.

Effect of low temperature on shootlets preservation

In this experiment the influence of low temperature on preservation of *in vitro* grown shootlets was investigated. Individual shootlets (1 cm length) were cultured on MS medium containing 1 mg/l BA and then incubated at complete dark at 5 °C for nine months. Number of proliferated shootlets, shootlets height (cm), number of branches and necrosis percentages were registered after three, six and nine months.

Culture medium and incubation conditions

The culture medium was supplemented with 3 % (w/v) sucrose and 7 g/l agar and adjusted to pH 5.7, with 25 ml in 200 ml capacity glass jars prior autoclaving at 121°C and 1.2 kg/cm^2 for 20 min. The cultures of osmoticums experiments were incubated under normal conditions i.e., 25 ±2 °C, 16 h photoperiod. However, cultures of the low temperature experiment were incubated at complete dark at 5 °C.

Experimental design and statistical analysis

Experiments were run in completely randomized design and data were statistically analyzed using standard error (SE) according to the method described by Snedecor and Cochran (1967).

Results and Discussion

Mannitol is used as an osmotic agent in plant tissue culture studies. The addition of mannitol to plant tissue culture medium decreases the water potential of the media inducing water stress that adversely affected the growth. This experiment was conducted to investigate the effect of three concentrations (20, 40 and 60 g/l) of mannitol on *in vitro* preservation of jojoba shootlets. The obtained results indicate that increasing the osmotic stress induced by mannitol caused a progressive reduction in both number of proliferated shootlets and shootlets height (Table 1). However, number of branches increased as mannitol increased in culture medium. The highest numbers of shootlets (6.50) and shootlet length (3.35 cm) were recorded with mannitol free medium. The lowest values of shootlets number and shootlets height were registered with 60 g/l containing medium. However, providing the conservation media with 60 g/l mannitol raised mean of branches number. The greatest number of branches was found under 60 g/l mannitol treatment with values of 3.20. On the other hand, necrosis (20 %) was noticed only when shootlets were preserved on medium contained 60 g/l mannitol (Fig.1-A). On the contrary, no necrosis was observed with other treatments.

Table 1: Effect of different concentrations of mannitol on *in vitro* preservation of jojoba shootlets.

Mannitol (mg/l)	Proliferated shootlets	Shootlets high (cm)	Numbers of branches	Necrosis (%)
0.0	6.50 ± 0.20	3.35 ± 0.17	2.40 ± 0.15	0.0
20	5.00 ± 0.25	2.90 ± 0.32	2.80 ± 0.30	0.0
40	3.50 ± 0.18	2.00 ± 0.23	3.00 ± 0.20	10
60	2.00 ± 0.34	1.60 ± 0.19	3.20 ± 0.37	20

Mean ± SE, n= 20.

Slow growth techniques, based on the manipulation of culture media allow cultures to remain viable with a slow growth rate. The addition of osmoticums or growth retardants to the medium has proved efficient for reducing growth rates of different plants species. In this respect, osmoticums such as mannitol or sorbitol reduce mineral uptake by cells through differences in osmotic pressures thereby retarding plant growth (Thompson *et al.,* 1986). Mannitol was reported to be one of the substances lengthening the storage life of *in vitro* grown plant tissues (Shibli *et al*., 2006). In the present study, osmolite used for growth reduction was mannitol in 20, 40 and 60 g/l concentrations. The behavior of short-term storage was evaluated after 9 months of maintenance. Our results showed the positive effect of mannitol on *in vitro* conservation of jojoba shootlet cultures. Mannitol decreased shootlets length at all used concentrations. Also mannitol at 20, 40 and 60 g/l decreased the number of newly formed shootlets. Our observations are in agreement with Gabr and Sayed (2010). They mentioned that the highest numbers of *Deutzia scabra* shootlets grown *in vitro* were recorded on control medium and medium containing 20 g/l mannitol after 3 months. The longest shootlets was recorded on medium without mannitol and with after 12 months. Fathy and Abd El-Kader (2012) studied the storage treatments affected number, length and quality of axillary shoots of *Balanites aegyptiaca*. They found that, more shoots, which were longer and of better quality, was obtained at control conditions as compared addition of osmatic substances. Moges *et al.* (2003) found that increasing the concentration of mannitol, decreased the elongation of explants during preservation period of African violet (*Saintpaulia ionantha* Wendl.). Our results also are partially in agreement with the findings of other researchers (Westcott *et al*., 1977; Xin 1988), who successfully used mannitol in different concentrations for slow growth conservation of potato and sweet potato germplasms, obtaining survival rates of 70%-100%. In this respect, mannitol, as an osmoticum in culture medium, facilitates satisfactory conservation of pear rootstocks (Lambardi *et al*. 2006). Poor response in shoots of apple genotypes was observed when medium was supplemented with higher concentrations (3% and 4%) of mannitol (Kovalchuk *et al*., 2009).

Effect of polyethylene glycol on shootlets preservation:

Retardation in growth in tissue-cultured grown plants exposed to osmotic stress has been achieved by addition of PEG. In this study, three (5, 10 and 15 g/l) concentrations of PEG were subjected in jojoba shootlets grown *in vitro* for slow growth storage. The response of *in vitro* cultured shootlets stored for 9 months to PEG containing medium was assessed on the basis of proliferated shootlets, shootlets height, number of branches and percentage of necrosis. Data obtained revealed that supplementation of culture medium with PEG significantly decreased the recorded growth parameters (Table 2). Otherwise, PEG sharply reduced the number of shootlets. The highest values of proliferated shootlets and number of branches were recorded on control (PEG-free medium). Also, the longest shootlets was recorded on medium without PEG. The growth parameters gradually decreased as increasing of PEG in culture medium. It is quite clear from Table (2) and Fig (1-B) that the lowest values of proliferated shootlets (1.50), shootlets height (2.50 cm) and number of branches (1.50) were registered with 15 g/l PEG. It is important to notice that in the presence of PEG, the growth values were more decreasing comparing to that registered in the presence of mannitol. Moreover, necrosis of shootlets was observed at the three PEG concentrations (Table 2). The highest percentage (30 %) of necrosis was recorded when culture medium was provided with 15 g/l PEG.

Table 2: Effect of different concentrations of PEG on *in vitro* preservation of jojoba shootlets.

PEG (g/l)	Proliferated shootlets	Shootlets high (cm)	Number of branches	Necrosis (%)
0.0	6.60 ± 0.15	3.30 ± 0.17	2.50 ± 0.19	0.0
5	3.50 ± 0.28	3.00 ± 0.24	2.45 ± 0.29	10
10	2.00 ± 0.18	2.70 ± 0.21	2.00 ± 0.32	25
15	1.50 ± 0.22	2.50 ± 0.31	1.50 ± 0.12	30

Mean ± SE, n= 20.

Polyethylene glycol (PEG) is the best known selective agents that increase the osmotic pressure in culture media and mimic drought stress without causing too many other physiological artifacts (Manoj *et al*., 2011). In our study, addition of PEG was an effective in growth reduction of jojoba shootlets grown *in vitro.* This may be due to the gradual increase in osmotic pressure in the medium, which resulted in reduced availability of water to the growing cultures. Comparing the influence of mannitol on jojoba growth, it could be suggested that PEG decreases growth more than mannitol. Similar responses have been observed by Piwowarczyk *et al.* (2014) who found that an increased concentration of PEG in the media caused a decrease in the multiplication rate and vigor of newly regenerated shoots of *Lathyrus sativus* grown *in vitro*. They mentioned that this may be a consequence of hampered water and nutrient absorption due to a decreasing water potential of the medium. Bekheet (2015) mentioned that PEG caused a progressive reduction in both fresh weight and growth value of callus cultures of Milk thistle. The effect of water stress induced by polyethylene glycol on callus growth, callus water content, callus necrosis and regeneration was also investigated on four cultivars of durum wheat (Bouiamrine and Diouri, 2012). The results showed that increasing PEG concentration in the medium causes a gradual decrease in growth and water content of callus. High concentrations of PEG caused callus necrosis as well. They considered callus necrosis percentage as an indicator of tissue culture intolerance to osmotic stress induced by osmotic stress agents such as polyethylene glycol PEG or mannitol.

Effect of low temperature on shootlets preservation:

Slow growth is usually achieved by reducing the incubation temperature, by modifying culture media with supplements of osmotic agents, growth inhibitors, or by removing growth promoters. In this experiment, possibility of short-term storage *in vitro* of jojoba shootlets using low temperature was evaluated. Shootlets were cultured on MS medium containing 1 mg/l BA and then incubated at 5 °C for 3, 6 and 9 months. The results of the different incubation periods were compared with the control (normal incubation temperature). Data obtained generally reveal that low temperature has tended to reduce both number of shootlets and shootlets height. However, low temperature obviously enhanced the regenerated branches of jojoba shootlets. Otherwise, the storage period also had a significant effect on number of shootlets and shootlets height (Table 3 and Fig. 1-C). The cultured shootlets stored for 9 months had the greatest decrease in both growth parameters. The highest number of shootlets (7.50) and shootlets height (4.50 cm) were recorded on control after nine months of incubation. Also, this treatment gave the

maximum percentage (40 %) of shootlets necrotic. On contrast, the highest number of branches (4.10) was obtained from incubation at 5 °C for nine months (Table 3). It important here to mention that no necrosis was observed with the three periods low temperature storage.

Table 3: Effect of low temperature incubation on *in vitro* preservation of jojoba shootlets.

Growth parameters	Three months storage		Six months storage		Nine months storage	
	Normal temperatur	Low temperature	Normal temperature	Low temperature	Normal temperature	Low temperature
Proliferated shootlets	6.55 ± 0.12	4.00 ± 0.22	7.20 ± 0.14	5.00 ± 0.10	7.50 ± 0.13	5.00 ± 0.16
Shootlets high (cm)	3.25 ± 0.21	3.20 ± 0.18	4.00 ± 0.19	3.30 ± 0.15	4.50 ± 0.25	3.30 ± 0.23
Numbers of branches	2.50 ± 0.1	3.40 ± 0.31	3.00 ± 0.21	4.00 ± 0.20	3.30 ± 0.27	4.10 ± 0.17
Necrosis (%)	0.0	0.0	10	0.0	40	0.0

Mean ± SE, n= 20.

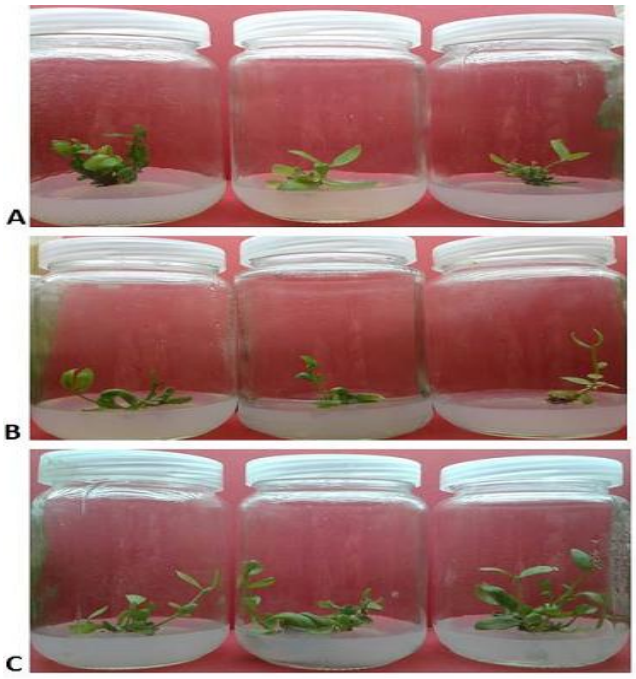

Fig. 1: Shootlets of jojoba preserved for nine months on medium contained (A) 20, 40 and 60 g/l mannitol from left to right and (B) 5, 10, 15 g/l PEG and (C) shootlets preserved at 5 °C for 3,6 and nine months from left to right.

In vitro conservation method largely depends on achieving sufficient growth limitation and maximal viability. Medium-term conservation by means of *in vitro* slow growth storage allows extending subcultures from several months to several years, depending on the species. In this context, storage under low temperature is one of the major tissue technique used for preservation of plant genetic resources. In the present study, a pronounced decline in growth parameters presented as number of shootlets and shootlets height of jojoba was observed following storage at 5 °C. However, this condition obviously enhanced number of the proliferated branches. The obtained results are in accordance with those reported by Bekheet *et al*. (2001) who reported that shoot buds and callus cultures of date palm were successfully stored for 12 months at 5 ºC in dark. Also, low temperature (2-10 °C) for minimal growth storage of cultured plant cells and organs has been applied successfully to apple (Lundergan and Janick, 1979). However, Hassanen (2013) mentioned that maintenance of Grapevine shootlets culture at 15°C showed positive affected for preservation. Similar response has been observed also by Tokoporo *et al*. (2013) who successfully conserved shoot-tip explants of banana at 15 ºC. In this respect, several reports have been published on *in vitro* storage of plant tissue cultured materials at low temperatures. Fortes and Pereira (2001) reported that the growth rate of micropropagated asparagus shoots maintained at 6 ºC for 4 months was 15.6% and the survival rate after 9 months, regardless of treatments, was 25%. Yu *et al*. (2003) stored 22 varieties of strawberry at 4ºC and reported that only 8 varieties could be preserved for 4 months with a survival

percentage of over 50%. Benelli *et al*. (2003) reported that grape shoots kept at 10 ºC had higher survival rate (80%) than those conserved at 4ºC.

Conclusion

Our study concerning the induction of *in vitro* minimal growth of jojoba proved that both mannitol and PEG can be used for short-term storage of shootlets cultures. In case of PEG, the limitation of growth is stronger but more shootlets necrosis was observed. Mannitol caused slight increasing of new branches induction. Otherwise, low temperature reduces both number of shootlets and shootlets height and obviously encouraged new branches regeneration. No necrosis was observed with low temperature until nine months storage. We can conclude that low temperature (5°C) is better for healthy storage of *in vitro* grown shootlets of jojoba.

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