Plant regeneration through direct and indirect somatic embryogenesis from immature seeds of citrus

Alam Ara Gholami[1]*, Seyed Vahid Alavi[2], Ahmad Majd[3] and Fathollah Fallahian[4]

[1]*Department of Biology, Science and Research Branch, Islamic Azad University, Tehran, Iran*
[2]*Plant Protection Division, Agricultural and Natural Resources Research Center of Mazandaran, Sari, Iran*
[3]*Department of Biology, Tehran North Branch, Islamic Azad University, Tehran, Iran*
[4]*Department of Biology, Science and Research Branch, Islamic Azad University, Tehran, Iran*

_____

ABSTRACT

In this study, the effects of various media on callus induction and direct and indirect somatic embryogenesis from immature seeds of Eureka lemon [Citrus limon (L.) Burm.f. ('Eureka')] was examined. Immature seeds were cultured on MS medium supplemented with the following compositions: N1 (500 mg l^{-1} malt extract + 30 g l^{-1} sucrose), N2 (500 mg l^{-1} malt extract + 50 g l^{-1} sucrose), and N3 (500 mg l^{-1} malt extract + 50 g l^{-1} sucrose + 3 mg l^{-1} BA). Maximum embryogenesis (direct and indirect), highest level of embryogenic callus induction, and embryo maturation were achieved in the N3 medium. The callus obtained in this medium was friable and creamy. The least embryogenic callus and non-maturity of embryos were observed in the N1 medium, and the callus obtained was compact and light green. Plantlets were regenerated from the embryos 2 months after their transfer to the germination medium without any growth regulators but with the addition of 50 g l^{-1} sucrose.

Keywords: Citrus, Immature seed, Direct somatic embryogenesis, Indirect somatic embryogenesis

_____

INTRODUCTION

Citrus is one of the most economically important fruit trees in the world. Somatic embryogenesis is an efficient method of plant regeneration allowing for rapid production of a large number of healthy plantlets within a short period. Recent studies have demonstrated major improvements in the development of efficient protocols for the *in vitro* regeneration of somatic embryos of citrus. Many studies on *in vitro* somatic embryo regeneration have been done [7, 11, 13]. However, few reports exist regarding somatic embryogenesis from somatic tissues without nucellar or ovular origin. Somatic embryogenesis mostly occurs indirectly through an intervening callus phase or directly from initial explants. In direct somatic embryogenesis, the embryo is formed directly from a cell or small group of cells without the production of an intervening callus. Direct somatic embryogenesis is generally rare compared with indirect somatic embryogenesis. Somatic embryos, embryogenic callus, and cell cultures recovered from *in vitro* cultured ovules have also been used to develop cryopreservation strategies for germplasm conservation [5, 14].

Malt is a most common medium supplement to induce *in vitro* embryogenesis in citrus [3]. However, embryogenesis has been enhanced by the addition of other growth substances such as 6-benzylaminopurine (BAP), casein hydrolysate, glycerol, lactose, and maltose [4, 8, 14]. The concentration of sucrose is critical to citrus callus induction and formation of the embryogenic callus [2]. BAP is an important plant growth regulator for the regeneration of citrus somatic embryos. The process of somatic embryogenesis is a suitable method for micropropagation and has the potential for use in the commercial propagation of healthy citrus at low per unit cost. In this study, the efficiency of direct and indirect *in vitro* embryogenesis from various types of somatic tissues of

_____

Eureka lemon [*C. limon* (L.) Burm.f. ('Eureka')] was studied on culture media with different supplemental growth regulator compounds and at various embryonic stages.

MATERIALS AND METHODS

Plant material
Eureka lemon [*Citrus limon* (L.) Burm.f. ('Eureka')] was used in this study. Immature fruits (10-14 weeks old) were collected from citrus orchards in east of Mazandaran province, Iran. These fruits were stored at 4°C until use. The fruits were thoroughly washed under running tap water three times to reduce surface contaminants and then surface sterilized by submerging in 70% (v/v) ethanol for 5 min, followed by 4% sodium hypochlorite solution for 10 min, and finally rinsed five times with sterile distilled water. Under a laminar airflow cabinet, the fruits were cut open using a sharp sterilized scalpel. The immature seeds were then separated and placed on the surface of the culture media.

Media and culture conditions
Three different media were used; MS (Murashige and Skoog, 1962) medium supplemented with 500 mg l^{-1} malt extract and 30 g l^{-1} sucrose (N1), MS supplemented with 500 mg l^{-1} malt extract and 50 g l^{-1} sucrose (N2), and MS supplemented with 500 mg l^{-1} malt extract, 50 g l^{-1} sucrose, and 3 mg l^{-1} BAP (N3). After adjusting the pH to 5.8 ± 0.1 using 1 M NaOH, 1% Difco Bacto agar was added to the media. The media were sterilized by autoclaving at 121°C for 15 min, and 25 ml medium was stored in 100-ml flasks and sealed with Parafilm (American Can, USA). The embryogenic calli were regularly subcultured on the same fresh medium every 21 days. Somatic embryos obtained from immature seeds were isolated and cultured in test tubes containing 15 ml solid MS medium with agar (8 g l^{-1}) supplemented with 50 g l^{-1} sucrose; this medium was hormone free. The culture tubes and flasks containing the explants were incubated in a culture room at 25 ± 1°C under 16-h day length with an illumination of 100 µmol m^{-2} s^{-1} provided by Osram cool white 18 W fluorescent lamps. Plantlets were stored for a year under the abovementioned conditions. Callus formation scoring was started 3 weeks after treatment initiation and was continued for 8 months at an interval of 10 days. Ten flasks were selected for each medium, and 10 immature seeds were selected from each flask.

Statistical analysis
The experiment was a completely randomized two-factor design with 20 replicates. The data were analyzed statistically using one-way analysis of variance, and the significant differences between means were assessed by Duncan's multiple range tests at $p < 0.05$ using the Statistical Package for Social Science (SPSS ver. 16).

RESULTS

Somatic embryos without an intermediate callus phase were directly induced on the surface of immature seeds 2 months after culture initiation on each medium. Our results show that the maximum efficiency of direct somatic embryo formation (49.50) occurred on the N3 medium. The minimum efficiency was 19.75. During indirect somatic embryogenesis, the callus is first obtained from immature seeds and then embryos were produced on the embryogenic callus**.** Embryogenic callus was observed 30 days after treatment initiation. Callus initiation occurred from immature seeds on all culture media after 3 weeks of culture. Embryos produced on the surface of the calli were observed 4 months after culture initiation in the different developmental stages. Duncan's post hoc test was used to detect differences between the factors (Table 1). The N3 medium had the least browning and most embryonic calli with indirect somatic embryogenesis. This medium also had the highest plantlet production, and the results for this medium were significantly different from those for the other media.

The 27.5%-68.8% indirect somatic embryogenesis and 22%-9.40% plantlet production was observed. The highest rates of direct embryogenesis (49.5%) were achieved for Eureka lemon in the N3 medium, making Eureka lemon and the N3 medium the best combination for direct embryogenesis. Rate of embryogenic callus induction in the N3 medium was 44.5%.

The color and texture of callus on different culture media were different. The N1 medium produced compact callus with light green colors. This medium produced the least embryogenic callus and the least embryos. Friable callus with a light green color was obtained on the N2 medium, and friable callus with a light yellow color was seen on the N3 medium (Table 2).

Significant differences ($p < 0.05$) were observed between different treatments and their effects on the rate of embryonic callus induction, embryo development, and regeneration of cotyledonary embryos into plantlets (Table 3). In the N1 medium (with malt extract and low sucrose concentration without the plant growth regulators),

embryogenic callus induction (9.87%) was lowest, and the embryos obtained from this medium developed to the initial stages of heart-shaped embryos. The rates of embryogenic callus formation and embryo maturation in the N1 medium were significantly reduced compared to the N2 and N3 media. In the N2 and N3 media, globular and heart-shaped embryos produced from embryogenic callus developed into cotyledonary embryos that turned into plantlets after being transferred to the germination medium. The rate of embryogenic callus formation in the N2 medium (38.7%) was significantly different (p<0.05) from that in the N3 medium (44.55%). The results demonstrated that the N3 medium was suitable for indirect somatic embryogenesis, embryo maturation, and torpedo-shaped and cotyledonary embryos. Induction was observed on the N2 and N3 media, but no torpedo-shaped and cotyledonary embryos were observed on the N1 medium.

The plantlets were regenerated two months after transferring cotyledonary embryos to the germination medium (MS medium supplemented with 50 g l^{-1} sucrose and no plant growth regulator). The rate of plantlet production in the N3 medium was 33.01%. The N2 medium had lower plantlet regeneration rates (30.4%), and no plantlets were regenerated in the N1 medium.

Table 1. Comparison of mean values using Duncan's multiple range test in three cultivation media for the rates of direct and indirect embryogenesis in two varieties of lemon and tangelo

Treatments	Browning rate	Direct embryogenesis	Indirect embryogenesis	Plantlet (%)
N3	1.00 ± 3.08^i	49.50 ± 1.54^b	49.50 ± 1.54^b	33.01 ± 1.03^b
N2	14.00 ± 1.59^h	43.50 ± 1.54^c	42.50 ± 2.19^c	30.40 ± 2.14^c
N1	60.50 ± 6.05^b	19.75 ± 3.02^f	19.75 ± 3.02^h	0.00 ± 0.00^i

Values are expressed as the mean ± SE of rate of direct and indirect embryogenesis. Means within columns with the same letter were not statistically different at p<0.05 using Duncan's multiple range test

Table 2. Differences in embryogenic callus obtained from immature seeds 3 weeks after culture initiation

Callus status	Embryogenesis and embryo maturation	Type of culture medium
Compact and light green	+	N1 (MS + 500 mg l^{-1} malt extract + 30 g l^{-1} sucrose)
Friable and light green	++	N2 (MS + 500 mg l^{-1} malt extract + 50 g l^{-1} sucrose)
Friable and light yellow	+++	N3 (MS + 500 mg l^{-1} malt extract + 50 g l^{-1} sucrose + 3 mg l^{-1}BAP)

(+) means less than 1%, (++) means between 1 and 30% and (+++) means more than 30%

Table 3. Comparison of mean values by Duncan's multiple range test in three culture media in different embryonic stages

Media	Somatic embryo stage development						
	Indirect embryogenesis (%)	Embryogenic callus (%)	Globular (%)	Heart (%)	Torpedo (%)	Cotyledonary (%)	Plantlet (%)
N3	49.50 ± 1.54^b	44.55 ± 1.39^b	42.54 ± 1.38^b	38.74 ± 4.20^b	35.65 ± 3.57^b	33.60 ± 1.79^b	33.01 ± 1.03^b
N2	42.50 ± 2.19^c	38.70 ± 3.08^d	36.50 ± 3.58^c	35.15 ± 3.65^c	33.70 ± 3.83^c	31.90 ± 3.43^c	30.40 ± 2.14^c
N1	19.75 ± 3.02^e	9.87 ± 1.51^e	5.93 ± 2.18^e	$3.12 \pm .63^d$	$.00 \pm .00^e$	$.00 \pm .00^e$	$.00 \pm .00^e$

Data are the mean values of 20 replications. Values are expressed as the mean ± SE. Means within columns with the same letter were not statistically different at p<0.05 according to the Duncan's multiple range test

DISCUSSION

In some species, somatic embryos were obtained from special explants cultured on hormone-free media [1, 16]. However, in the present study, the best embryogenesis (direct and indirect) was obtained in the medium supplemented with high sucrose and BAP. Eureka lemon presented a good response in terms of direct somatic embryogenesis. Therefore, the combination of genotype and culture medium plays a vital role in somatic embryogenesis induction in citrus fruits. Plants from various genotypes that possess different internal hormonal balances show different reactions to stimulants that induce somatic embryogenesis [9, 12]. Different conditions of callus could indicate the ability of this callus to cause induction of embryogenesis and maturation of embryos from the callus. These results correspond to the findings of Lincy et al. [10] who reported that the texture and color of embryogenic and non-embryogenic callus are different. Our results demonstrated that friable callus with a light yellow color have high potential to induce embryo maturation (N3 medium), while compact callus with a light green color did not have the same potential to induce development of embryos into more advanced stages (torpedo and cotyledonary) (N1 medium). A stress factor appears to be needed for embryogenic callus induction and somatic embryo formation. Our results demonstrated that sucrose as a stress factor can cause embryogenic callus induction and somatic embryo maturation. These results were similar to the findings of Lincy et al. [10]. The present study indicated that the N1 medium with low sucrose concentration (30 g l^{-1}) combined with malt extract (500 mg l^{-1}) has a low potential for callus induction and cannot cause embryo maturation (development into torpedo-shaped and cotyledonary embryos). However, media with high sucrose concentration (50 g l^{-1}) combined with malt extract (500 mg l^{-1}) showed considerable potential for callus induction and embryo maturation. Increasing sucrose concentration from 30 to 50 g l^{-1} significantly increased embryogenic callus induction, development of heart-shaped embryos, and

309

_____

embryo maturation. Increased sucrose concentration is probably associated with osmotic changes and these osmotic stresses play an important role in somatic embryo maturation [6].

Embryo maturation is dependent on both sucrose concentration and presence of BAP. The results indicated that the medium containing high sucrose concentration and BAP had a positive effect on embryo maturation (N3 medium), which was significantly different from the effect of the medium with high sucrose concentration and no BAP (N2 medium). In the N2 medium, embryo maturation was significantly reduced. The results were similar to the findings of Carimi [2] who also demonstrated that high sucrose concentration along with BAP plays an important role in somatic embryogenesis induction in citrus fruit. It can therefore be deduced that cells of the seed tissues may have been predestined for embryogenesis and presence of regulators such as BAP stimulates somatic embryo production and embryo maturation.

The results indicated that the best medium for embryogenic callus induction, embryo maturation, and regeneration of plantlets from immature seeds is a medium with malt extract, high sucrose concentration (50 g l^{-1}), and BAP. The embryos obtained through this method could be used as a raw material for the production of synthetic seeds of these plants.

REFERENCES

[1] N. Burbulis, R. Kupriene, *Acta Univ. Latv*., **2005**. 691, 137-143.
[2] F. Carimi, *Options Mediterraneennes Serie B*, **2001**. 33, 115-128.
[3] F. Carimi, *Springer*, **2005**. pp. 321-343.
[4] F. Carimi, M.C. Tortorici, F. De Pasquale, F.G. Crescimanno, *Plant Cell Tiss. Org. Cult*., **1998**. 54, 183-189.
[5] F. Engelmann, D. Dambier, P. Ollitrault, *Cryo-Lett*., **1994**. 15, 53-58.
[6] M.D. Gaj, *Plant Growth Regul*., **2004**. 43, 27-47.
[7] M.A. Germana, *Biologia*, **2003**. 58, 843-850.
[8] Y.J. Hao, C.X. You, X.X. Deng, *J. Plant Physiol*., **2004**. 161, 479-484.
[9] V.M. Jimenez, *Revista Brasileria De Fisiologia Vegeta*, **2001**. 13, 196-223.
[10] A.K. Lincy, A.B. Remashree, B. Sasikumar, *Acta Botanica Croatica*, **2009**. 68, 93-103.
[11] N. Nito, M. Iwamasa, *Plant Cell Tiss. Org. Cult*., **1990**. 20, 137-140.
[12] R. Pereze, A.M. Galiana, L. Navarro, N. Duran-Vila, *J. Hortic. Sci. Biotech*., **1998**. 73, 796-802.
[13] H. Rong, R.J. He, S.C. Chen, J.R. Zhang, *South China Fruits*, **1998**. 27, 9-10.
[14] A. Sakai, S. Kobayashi, I. Oiyam, *Plant Cell Rep*., **1990**. 9, 30-33.
[15] M.L. Tomaz, B.M.J. Mendes, F. Assis, F. Filho, C.G.B. Demetrio, N. Jansakul, A.P.M. Rodrogues, *In Vitro Cell. Dev. Biol.-Plant*, **2001**. 37, 446-452.
[16] H.C. Wu, E.S. Toit, C.F. Reinhardt, *Plant Cell Tiss. Org. Cult*., **2007**. 89, 217-222.