A study on evening-primrose (*Oenothera biennis* L.) callus regeneration and somatic embryogenesis

Azim Ghasemnezhad*, Seyyed Javad Mousavizadeh, Kambiz Mashayekhi

Department of Horticultural Sciences, Gorgan University of Agricultural Sciences and Natural Resources, P.O. Box 49138-15739, Gorgan, I.R. Iran

Abstract

Evening primrose (*Oenothera biennis* L.) is a wild flower with high and valuable oil content. High seed shattering, indeterminate inflorescence and heterogeneous germination limit the commercial cultivation of this plant. Besides agronomical research, breeding programs can also remedy the above mentioned problems. Since traditional breeding methods take a long time, using techniques such as tissue culture and somatic embryogenesis accelerate the breeding process. In the present study, callus formation was accomplished in MS (Murashik and Skoog) medium, but no embryogenesis was observed in the presence or absence of plant hormones like 2,4-D (2,4-dichlorophenoxy) acetic acid. In contrast, B5 (Gamborg) medium containing 2,4-D induced embryogenesis. Different parts of plants exhibited good callus production potency and the hypocotyl was found as the best plant explants. In B5 medium, various concentrations of 2,4-D (0,2.26 μM, 4.52 μM, and 9.04 μM) were applied as a complete randomized design experiment with 4 replications. For embryogenesis, hypocotyls (1cm long) were cultured in B5 liquid medium at the induction phase. After 3 weeks, induced organs were sub-cultured to realization phase and the number of embryos (different stages of embryogenesis) was counted 4 weeks later. The results indicated that variation in hormone concentrations caused significant differences with respect to somatic embryogenesis. Embryo development were not observed in hormone-free media. The highest numbers of globular, heart, torpedo, cotyledonary, and total embryo was recorded at 9.04 μM of 2, 4-D. Histological studies of embryos after the realization phase revealed large nuclei and abundance of starch grains indicating the presence of embryonic cells in evening primrose hypocotyls.

***Key words*:** Evening primrose, Gamma linolenic acid, Medicinal plant, Tissue culture, Somatic embryogenesis

Correspondence to:* **Azim Ghasemnezhad, Ph.D.
Tel: +98 171 4423303; Fax: +98 171 4420981
E-mail: ghasemnezhad@gau.ac.ir

INTRODUCTION

Evening primrose (*Oenothera biennis* L.) is a biennial plant which is cultivated as an annual crop (Ghasemnezhad and Honermeier, 2007). It is an oil seed crop which due to the presence of gamma linolenic acid (GLA) an unsaturated fatty acid has noticeable pharmaceutical and nutritional values (Mendoza *et al*., 2005; Murphy *et al*., 2004). Rheumatic arthritis, breast pain, skin disorders like eczema, and alcohol disorders are positively influenced by evening primrose seed oil (Huang and Ziboh 2000; Kris-Etheroton *et al*., 2000). The increasing market for this plant has given producer the opportunity to replace food crops by non-food crops (Fieldsend and Morison, 2000). Despite the presence of higher levels of GLA in seeds of plants such as black currant (*Ribes nigrum*), borage (*Borago officinalis*) and the oil produced by some species of the fungus *Mucor* genus (Lapinskas, 1997), evening primrose oil appears to have the most biologically active form of GLA (Peschel *et al*., 2007; Barre 2001; Shewry *et al*., 1997; Wolf *et al*., 1983). Special composition of fatty acids in triacylglycerol molecules makes the GLA of evening primrose oil easily accessible to hydrolysis by pancreatic lipase in the small intestine (Rahmatullah *et al*., 1994). Although evening primrose has a good

potential to become a commercial agricultural crop for the production of GLA, but some disadvantages, such as indeterminate inflorescence, high seed shattering during ripening and heterogeneous germination could result in significant impediments. Despite all attempts to decrease the seed-shattering characteristic, it is still a major problem in the production of evening primrose (Simpson and Fieldsend, 1993). Although agronomical technique such as consideration of optimum harvesting time and appropriate harvest method increase seed yield by reducing seed shattering (Ghasemnezhad and Honermeier, 2007), breeding new cultivars with determinate growth behavior and low shattering is one strategy to overcome disadvantages of this plant. Since traditional breeding methods take a long time, biotechnological techniques such as tissue culture via somatic embryogenesis accelerate the breeding process. In fact, few researches have been carried out in this connection (Ghasemnezhad and Honermeier 2007; Pavingerova *et al*., 1996; Martinez and Noher De Halac 1995). Hence the goal of present study was to investigate the potency of callus formation and somatic embryogenesis of evening primrose.

MATERIALS AND METHODS

The present research was focused on evening primrose callus formation and somatic embryogenesis. Preliminary studies were conducted to see the callus formation potency of evening primrose tissue in the presence of 2,4-D dissolved in basic salts MS medium. Table 1 shows different treatments for callus generation in evening primrose tissues.

Explants were prepared from sterilized seeds germinated in ½MS medium. Full ripened seeds were treated with ethanol (70% v/v) for 30 s. Surface sterilization was then carried out by immersing in sodium hypochlorite (3% v/v) containing 2 drops of Tween 80, for 20 min. Sterilized seeds were subsequently germinated in ½MS containing 5g/l of sucrose and 2.2 g/l of Gelrite at a pH of 5.8. For callus formation, depending on the size of seedling, 2 to 4 weeks after germination, explants (1 cm in length) were prepared from different parts of the plant (Table 1), and were transferred to MS medium containing different levels of 2,4-D (Pinto *et al*., 2008). Study on somatic embryogenesis in both solid and liquid MS media was conducted using different levels of 2,4-D and kinetin (at the concentrations of 0.01, 0.1, 0.5, 1 ppm of 2, 4-D and kinetin, in single and combined forms). In the experiment that used B5 liquid medium for embryo induction, 4 explants (hypocotyl) were transferred to a T form balloon filled with 25 ml of the medium containing 0, 2.26, 4.52 and 9.02 µM 2, 4-D. The medium also contained 2% sucrose and 250 mg/l of casein hydrolyzate (Pinto *et al*., 2008), and its pH was adjusted to 5.7 using 0.1 N NaOH and HCl. The samples were kept on an Auxophyton apparatus rotating at the 1.9 rpm under 28ºC and permanent light (2000 luxe). Depending on medium and explant conditions subculturing was carried out every 3 weeks. Three weeks after the initiation phase (B5 medium containing 2,4-D) explants were entered to realization phase. Before transferring to the realization medium, samples were washed 3 times

Table 1. Study on callus formation of evening primrose.

Medium	PGR* (ppm)		Tissues	
0.01	Hypocotyl		0.10	Petiole
	0.50	Root		
	1.00	leaf		

Room temperature, 16 h light (2500 luxe cold florescent), plant growth regulator.

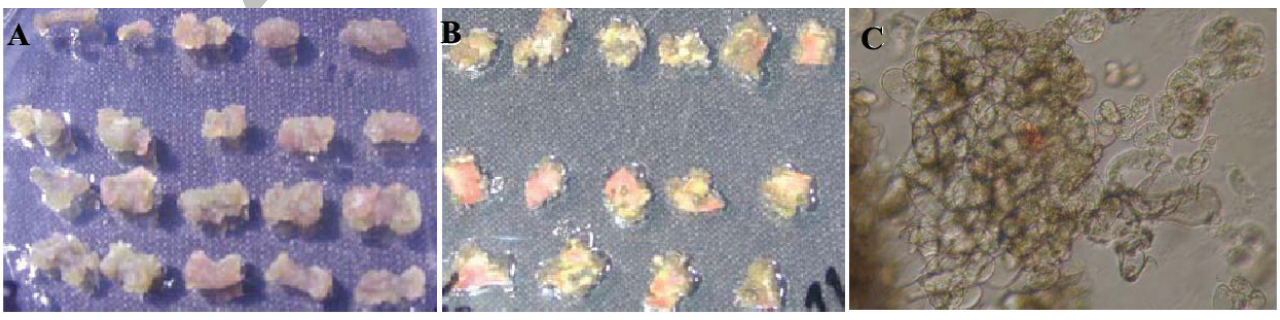

Figure 1. A: Callus regeneration of evening-primrose B: the hypocotyl C: the leaf in MS medium containing 0.1 ppm 2,4-D (two weeks after culturing), cell mass production in MS medium containing, 0.1 ppm 2,4-D and 0.01 ppm kinetin which was also supplemented with asparagine and glutamine.

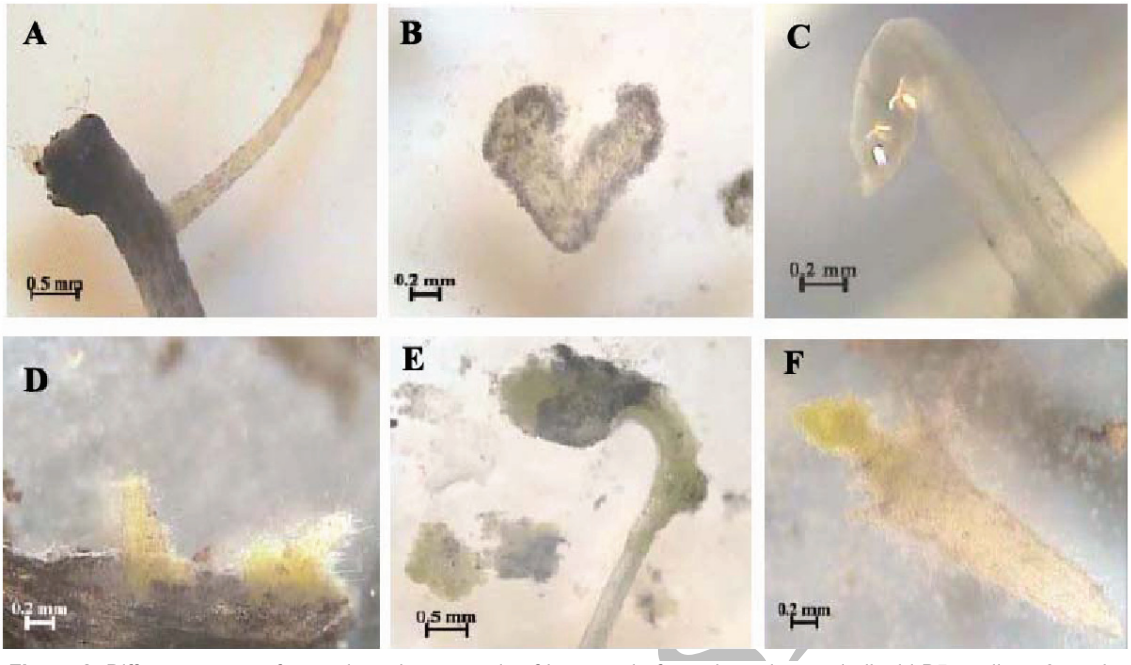

Figure 2. Different stages of somatic embryogenesis of hypocotyl of evening-primrose in liquid B5 medium, 3 weeks after the realization phase. A: roots in hormone-free medium, B: heart-shape embryo in medium containing 9.02 μM 2,4-D, C: primary stages of cotyledonary embryo formation, D: torpedo embryo releasing from the epidermis, E: neumorphe, F: cotyledonary embryo at cotyledon opening stage in B5 medium containing 4.25 μM 2,4-D.

with auxin-free medium at 5, 10 and 15 min intervals. Four weeks after the appearance of embryos, the number of vegetative embryos at the globular, heart, torpedo and cotyledonary stages was counted. Photos were taken using a computer connected to a stereo microscope (Sunny group ST60-24T2, Japan with×20 and ×40 magnifications). To confirm the formation of embryos, histological and microscopic studies also performed. Samples were fixed in a fixator solution containing glacial acetic acid, ethanol, and formalin at a ratio of 5:10:5 (v: v: v), respectively. Then diffusion and replacement of paraffin in the tissue was performed as follows: samples with a cross-section of 14 micron size were prepared using a microtome (Leitz 1212, Germany). At the deparafinization stage paraffin was removed from the tissues using xylene. Double staining of sections was carried out using safranin and light green. After staining, the sections were kept on slides using antalen and covered by cover slips (Ruzin 1999). Pictures were taken using microscope (Olympus BX51, Japan) connected to camera (Olympus DB12, Japan). The acquitted data were normalized using the squared root transformation procedure. Data analysis was then performed as a completely randomized design with four replications. Statistical analysis was carried out using SAS software (2001) and the least significant difference of mean values at 5% probability (LSD) was computed.

RESULTS

Explants such as the hypocotyl, petiole, and leaf were capable of producing callus. Nevertheless, callus formation varied based on the type of tissue and 2,4-D concentrations (Fig. 1 A). When leaf was used as an explant there was little callus formation (Fig. 1 B). Formation and growth of callus were strongly related to the concentration 2,4-D. As the concentration was increased from 0.0 to 0.1 ppm, callus formation as well as its growth rate increased. Although the mass of callus was increased with higher concentrations of 2,4-D (more than 0.1 ppm), but the resulting callus increased relatively smaller than the callus produced in medium containing 0.1 ppm 2,4-D. Besides the size, the color of the callus was also influenced by hormone levels, so that at the higher concentrations, its color turned from a yellowish to brownish green and dark brown. In the present study, several trials were carried out on somatic embryogenesis of evening primrose in solid and liquid MS media using concentrations of hormone as described in the Material and Method section.

Table 2. Variance analysis of different stages of embryo development of evening primrose.

Sources	df	GE	HE	TE	CE	TE
Treatment	3	9.73**	2.91**	11.19**	47.47**	61.1**
Error	12	0.03	0.04	0.04	0.47	0.49
CV%		6.66	16.81	10.38	20.24	13.68

(p<0.01)**, GE: globular embryo, HE: heart-shaped embryo, TE: torpedo embryo, CE: cotyledonary embryo, TE: total embryo.

Table 3. Mean value of somatic embryos of evening primrose based on LSD test (5%).

2, 4.D (μM)	GE	HE	TE	CE	TE
0.00	0^{c}	0^{c}	0^{c}	0^{c}	0^{c}
2.26	15.7^{a}	0^{c}	0^{c}	0^{c}	15.7^{c}
4.52	9.0^{b}	1.3^{b}	4^{b}	18.3^{b}	32.7^{b}
9.02	15.7^{a}	6^{a}	17.7^{a}	63.7^{a}	103^{a}
LSD 5%	3.5	0.034	3.7	15.8	12.4

GE: globular embryo, HE: heart-shaped embryo, TE: torpedo embryo, CE: cotyledonary embryo, TE: total embryo.

Generally, the results of embryogenesis using the MS medium showed that, on solid medium in all cases, no embryogenesis was observed, but in the liquid medium especially when glutamine (112.8 mg/l) and asparagine (0.068 mg/l) were added to media containing different concentrations of 2,4-D and kinetin, cell division and the number of cell masses increased significantly (Fig. 1 C). However, no embryo was detected at any of the stages. Table 2 shows the results of embryogenesis of evening primrose cells when B5 medium was used for indirect embryo induction without callus production. Based on these results, the number of formed- embryos was changed when different levels of the hormones was used. The highest number of embryos (63.67) was observed in those samples treated with 9.02 μM 2.4, D (Table 3). Results revealed that in samples which received 2.26 μM 2,4-D, only the globular embryo were observed. Among the hormone concentrations used, the smallest number of embryos (only undeveloped embryos) was observed in samples treated with 2.26 μM 2,4-D. Since the study of the plant tissues leads to a better understanding of plant conditions, histology played an important role in this regard. In Figure 3, a cross section of the evening primrose's hypocotyl was shown 30 days after the realization stage.

DISCUSSION

The results of the present study show that, with evening-primerose the hypocotyl tissue is the most suitable explant for callus formation and somatic embryogenesis. The finding in which the size and color of callus was influenced by the kind and the level of plant hormones is in accordance with the results of Ghauri and his colleagues (2008), who found that excellent production of callus biomass was achieved on MS medium supplemented with 2,4,-D. The absence of embryogenesis in MS medium that consisted of various hormones at different concentrations and additive substances suggested that this medium is not a suitable for callus embryogenesis in evening primrose. The use of B5 medium resulted in direct embryo induction without callus production. During direct embryo induction, cells which basically had the ability to undergo embryogenesis were affected by the hormones. Such a situation mostly appeared in young cells like those of the cells of meristem tissues (George *et al*., 2008). Along with the increase in the concentrations of hormone, an increase in the number of embryos at different stages of development was also observed. In fact, it has previously been observed that the number of developed embryos increased at higher hormonal concentrations. The effect of different concentrations of 2,4-D on somatic embryogenesis has been reported (Rai *et al*., 2007; Strosse *et al*., 2006). The results obtained by other researchers regarding embryogenic tissues, confirmed that, the signal transduction and preparation of the main conditions for the induction of embryogenesis is related to hormones and stresses which received by cells in tissues (Mizukami *et al*., 2008; Rai *et al*., 2007; Zouine *et al*., 2007; Strosse *et al*., 2006; Raghavan 2004). Thus by adding auxin especially 2,4-D to culture media, the direction of somatic cell differentiation can be changed, and totipotent cells divert to the formation of the embryos. The pre-mechanism of the effect of auxin on embryogenesis involves combination both of auxins and proteins (Ribnicky, *et al*. 1991). In an experiment on cell culture of carrot, Ribnicky *et al*. (1991) showed that, adding 2,4-D to the medium strongly increased the free IAA and its ester conjugated form. Thus, it can be concluded that 2,4-D induces the biosynthesis of IAA from tryptophan and related amino acids (Mashayekhi, 2007). In the present study, it was observed that some embryos did not reach the cotyledonary stage and remained as the undeveloped form, which is known as the neumorphe (Mashayekhi, 2007). Neumorphs are induced in culture media but are not released as com-

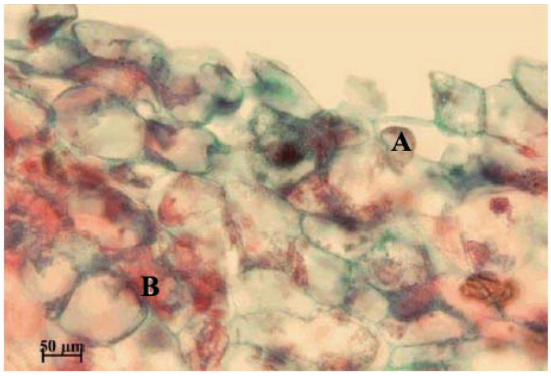

Figure 3. Cross section of the hypocotyl of evening primrose 30 days after the realization phase, in B5 medium containing 9.02 µM 2,4-D. A: represents the big nucleus of meristem tissues and B: shows the location of starch granules.

plete somatic embryos. For the development of neumorphs, it seems that some other treatments are necessary. In this regard Kordestani and Karami (2008) have shown that when undeveloped embryos of the strawberry plant were transferred to medium containing 1 and 2 ppm gibberellic acid (GA3), the number of developed embryos was significantly increased. In a study by Husaini *et al*. (2008), the highest number of developed strawberry embryos was developed when 1 ppm kinetin was added to the culture medium. In present study, which used the MS medium, when the explants were transferred to a medium containing 2,4-D and kinetin, cell division was observed, however, in the absence of kinetin rooting only occured. Thus, it is recommended that beside 2, 4-D, cytokinins and/or GA3 can be added to the medium, in order to obtain a higher number of developed embryos. The large nucleus in epidermal cells and the abundance of starch could be the sign of embryogenic tissues as reported by other researchers (Mashayekhi, 2007, George *et al*., 2008) and also showed in Figure 3. Generally the result of cell histology showed that the change has happened from normal to embryonic cells.

CONCLUSIONS

The results of the present study showed that evening primrose is a good plant for tissue culture. The potency of callus regeneration is good, and it seems that MS medium is the best. But with respect to somatic embryogenesis, it was found that the kind of medium and its composition play an important role. It has been shown that although 2,4-D directly stimulates the conversion of non-embryonic cells to embryonic ones, but in most cases additional hormones like cytokinin or gibberellin are needed to reduce the undeveloped embryos. Thus, further studies focused on other kinds of auxin and the combination of cytokinin and gibberellin with auxin are recommended to optimize an easy protocol for evening primrose somatic embryogenesis.

References

Barre ED (2001). Potential of evening primrose, borage, black currant and fungal oils in human health. *Ann Nutr Metab*. 45: 47-57.

Fieldsend AF, Morison JIL (2000). Climatic conditions during seed growth significantly influence oil content and quality in winter and spring evening primrose crops (*Oenothera* spp.). *Ind Crops Prod*. 12: 137-147.

George EF, Hall MA, Klerk GD (2008). Plant propagation by tissue culture. Springer. 3rd Edition. PP. 335-354.

Ghasemnezhad A, Honermeier B (2007). Seed yield, oil content and fatty acid composition of *Oenothera biennis* L. affected by harvest date and harvest method. *Ind Crops Prod*. 25: 274-281.

Ghauri EG, Shafi N, Fatima A,Ghani S (2008). High frequency plant regeneration from leaf explants derived callus of evening primrose (*Oenothera biennis*). *Pakistan J Biochem Mol Biol*. 41: 158-161.

Huang YS, Ziboh VA, (2000). *Gamma linolenic acid*: recent advanced in biotechnology and clinical applications. AOCS Press, Champaign, IL. PP. 259.

Husaini MA, Aquil S, Bha M, Qadri T, Kamaluddin AZM (2008). A high-efficiency direct somatic embryogenesis system for strawberry (*Fragaria ananassa* Duch.) cultivar chandler. *Crop Sci Biotech*. 11: 107-100.

Kordestani KG, Karami O (2008). Picloram-induced somatic embryogenesis in leaves of strawberry (*Fragaria ananassa* Duch.). *Acta Biologica Cracoviensia*. *Series Botanic*. 50: 69-72.

Kris-Etherton PM, Taylor DS, Yu-Poth S, Huth P, Moriarty K, Fishell V, Hargrove RL (2000). Polyunsaturated fatty acids in the food chain in the United States. *Am J Clin Nutr*. 71: 179-188.

Lapinskas P (1997). Production and marketing of GLA-containing oils. Proceedings of the International Symposium on New Approaches to Functional Cereals and Oils China, PP. 636.

Martinez LD, Noher De Halac (1995). Organogenesis of anther-derived Calluses in long-term cultures of *Oenothera hookeri* de varies. *Plant Cell Tissue Organ Cult*. 42: 91-96.

Martinez LD, Noher De Halac (1997). Callus induction in culture of *Oenothera hookeri* and *Oenothera picensis* anthers. *Biol Plant*. 40: 11-16.

Mashayekhi K (2007). Plant Somatic Embryogenesis. Makhtomgholi Fraghi (Sarli) press. P. 488.

Mendoza De Gives E, Sparks CA, Fieldsend AF, Lazier PA, Jones HD (2005). High frequency of adventitious shoot regeneration from commercial cultivars of evening primrose

(*Oenothera* spp.) using tidiazeron. *Ann Appl Biol*. 138: 329-332.

Mizukami M, Takeda T, Sayonara H, Matsuoka H (2008). Improvement of propagation frequency with two-step direct somatic embryogenesis from carrot hypocotyls. Biochemical Eng. 38: 55-60.

Mousavizadeh SJ (2009). The investigation of Strawberry and Carrot petiole explants behavior has been excised *in vitro*. Gorgan University of Agricultural Science and Natural Resources, Iran. *MSc thesis*. P. 108.

Murphy CL, McKenny CB, Auld DL, Hopper NW (2004). Field production of texas native evening primrose (*Oenothera* spp.) as a source of gamma linolenic acid. *Acta Hortic*. 629: 283-288.

Pavingerova D, Galis I, Ondrej M (1996). Tissue culture and transformation of *Oenothera biennis*. *Biol Plant*. 38: 27-32.

Peschel W, Dieckmann W, Sonnenschein M, Plescher A (2007). High antioxidant potential of pressing residues from evening primrose in comparison to other oilseed cakes and plant antioxidant. *Ind Crops Prod*. 25: 44-54.

Pinto G, Silva S, Yill-Sung P, Neves L, Araújo C, Santos C (2008). Factors influencing somatic embryogenesis induction in Eucalyptus globulus Labill.: basal medium and anti-browning agents. *Plant Cell Tissue Organ Cult*. 95: 79-88.

Raghavan V (2004). Role of 2, 4-Dichlorophenoxyacetic acid (2, 4-D) in somatic embryogenesis on cultured zygotic embryos of Arabidopsis: cell expansion, cell cycling, and morphogenesis during continuous exposure of embryos to 2,4-D. *American J Botany*. 9: 1743-1756.

Rahmatullah MS, Shukla VKS, Mukherjee KD (1994). Enrichment of λ- Linolenic acid from evening primrose oil and Boreg oil via lipase-catalyzed Hydrolysis. *J Am oil chem Soc*. 71: 569-573.

Rai MK, Akhtar N, Jaiswal VS (2007). Somatic embryogenesis and plant regeneration in *Psidium guajava* L. cv. Banarasi local. *Sci Hortic*. 113: 129-133.

Ribnicky DM, Michalczuk L, Cohen JD, Cooke TJ (1991). Auxin relationships during carrot somatic embryogenesis. 14th Int. Conf. Growth Substances. Amsterdam. Abstract.

Ruzin SE (1999). *Plant micro technique and microscopy.* University of California. ISBN 0-19-508956-1 (pbk), Oxford University Press Inc. PP. 307.

Shewry PR, Napier JA, Sayanova O, Smith M, Cooke DT, Stoker G, Hill J, Stobart AK, Lapinkase P (1997). *Domestication, production, and Utilization of new crops*. International Center for Under-utilised Crops, Southampton. PP. 76- 87.

Simpson MJA, Fieldsend AF (1993). Evening primrose harvest methods and timing. *Acta Hortic*. 331: 121-128.

Strosse H, Schoofs H, Panis B, Andre E, Reyniers K, Swennen R (2006). Development of embryogenic cell suspensions from shoot meristematic tissue in bananas and plantains (*Musa* spp). *Plant Sci*. 170: 104-112.

Wolf RB, Kleiman L, England RE (1983). New sources of λ-linolenic acid. *J Am Oil Chem*. 60: 1858-60.

Zouine J, Hadrami IE (2007). Effect of 2,4-D, glutamine and BAP on embryogenic suspension culture of date palm (*Phoenix dactylifera* L.). *Sci Hortic*. 112: 221-226.