

## **EMBRYOGENESIS AND RHIZOGENESIS IN MATURE ZYGOTIC EMBRYOS OF OLIVE (*OLEA EUROPAEA* L.) CULTIVARS *MISSION* AND *KRONEIKI***

M. Payvandi<sup>1</sup>, A. Dadashian<sup>2</sup>, H. Ebrahimzadeh<sup>3\*</sup> and A. Madjd<sup>4</sup>

<sup>1</sup> Department of Biology, Science and Research Unit, Islamic Azad University, Tehran, Islamic Republic of Iran

<sup>2</sup> Department of Industrial Crop, Ministry of Agriculture, Tehran, Islamic Republic of Iran

<sup>3</sup> Department of Biology, Faculty of Science, University of Tehran, Tehran, Islamic Republic of Iran

<sup>4</sup> Department of Biology, Faculty of Science, Tarbiat Moalem University, Tehran, Islamic Republic of Iran

### **Abstract**

Organogenesis and neoforment plantlet production were studied by culturing the different parts of mature embryo including radicle and proximal and distal parts of cotyledon of *Mission* and *Kroneiki* cultivars. Different parts of embryos were cultured in OMc medium containing growth regulators, such as indole butyric acid (IBA) and 2-isopentenyl adenine (2-ip) or IBA and naphthalene acetic acid (NAA). 21 days old calli were subcultured in medium without growth regulators or with auxin (IBA and NAA). Cultures were maintained in dark or 16h light/8h dark. Differentiation in the explants of *Mission* and *Kroneiki* cultivars depended on culture conditions. In both cultivars, the potentiality of embryogenesis in calli from radicle was more than calli from proximal, and in the latter was more than calli from distal parts of cotyledons. In addition, somatic embryogenesis was higher in dark. Subcultures of different parts in medium with auxin inhibited the embryogenesis. Rhizogenesis occurred in subcultures medium with or without auxin and in different photoperiods. In both cultivars the potentiality of rhizogenesis in calli from radicle and calli from different parts of cotyledon were observed. Differences between rhizogenesis from different cultivars and different subculture media were significant.

### **Introduction**

Olive tree (*Olea europaea* L.) has a high economic value and many countries such as Iran and Mediter-

**Keywords:** *Olea europaea* L.; cv. *Mission*; cv. *Kroneiki*; Somatic embryogenesis; Rhizogenesis  
rranean countries use its oil and conserved fruits [6,12]. Fashionable methods in plant breeding have not been

successful for this plant, but using biotechnology and *in vitro* plant regeneration may provide profitable results [2,11,16]. So far, olive organogenesis from mature and immature tissues has been reported to be successful. There are several reports about shoot regeneration from hypocotyl [3] and cotyledon segments [4,5]. Unfortunately, suitable methods of Micropropagation with axillary bud stimulation were possible only for few

\* E-mail: mpvandi@hotmail.com

olive cultivars [17,20]. But it has been reported that there were successful in embryogenesis from immature tissue [6,8,9,12,14,15, 18] and mature tissues [19].

The aim of these experiments is the micropropagation of two olive cultivars by somatic embryogenesis method. In this report we presented embryogenesis and rhizogenesis from radicle and cotyledon calli of mature embryos.

## Materials and Methods

### Plant Source

Two olive cultivars, well-known for their quality of oil production, were used. The fruits of *Mission* cv. are used for oil as well as conservation and the fruits of *Kroneiki* cv. are used for oil.

Fruits were collected in the middle of November until early January from Aliabad Olive Garden (*Mission* cultivar) and Agricultural Department Garden of Golestan province (*Kroneiki* cultivar). After harvesting, mesocarp was removed and seeds were dried after washing in room temperature. Experiments were begun in February and March in the same year.

### Isolation and Culture of Different Parts of Embryo

Just before beginning to culture, hard endocarp of seeds were mechanically broken and the surface of the seeds was sterilized with Ethanol 70% (1 min) and calcium hypochlorite 5% (20 min) and washed with sterile distilled water (5 times). Sterilized seeds were placed on sterilized glass petri dishes with two sterilized filter paper and 5 ml sterilized water were added to each petri dish. The petri dishes were maintained about 48 h in dark and in 24±2°C and then embryos were isolated from endosperm with scalpel. Radicle, proximal and distal parts of cotyledon were separately cultured in each petri dish (three explants in each petri dish).

### Medium

Three kinds of medium were used. For callus production from explants, OMc medium [5], with IBA (5 mg/l), 2-ip (0.5 mg/l) (medium I) and/or OMc medium with IBA (1 mg/l) and NAA (1 mg/l) (medium II) were used. Subcultures were then performed in OMc without hormones (medium III), and if the second medium was used, subcultures were performed in the same medium. In the three media the concentration of organic compounds (myo-inositol, glycine, thiamine HCl, pyridoxine HCl, nicotinic acid, biotin and folic acid) were 10 times higher than the one suggested by Rugini [17]. Somatic embryogenesis and rhizogenesis in each experiment (11 replicates) were expressed as the mean number of calli forming one or more embryos or roots. Analysis of variance was performed by factorial

design arranged in CRD (Completely Randomized Design) with transformed data (square root of data+1). Comparisons of means was then performed with Duncan test.

All experiments were conducted in a culture room with a temperature of 24±2°C and in dark or 16 h light/8 h dark photoperiod.

### Histological Observation

After the taking embryogenic tissues out of the medium, they were fixed for about 18 h in ethanol, acetic acid, formaldehyde (95:5:5) fixator, and were washed for 18 h under water current. Then dehydration and molding were performed in paraffin and slices from samples (10 µm) were prepared. After hydration, the samples were stained with haematoxylin (20 min) and eosine (5 min).

## Results

### Somatic Embryogenesis

(A) *Mission* cultivar. 21 day-old calli obtained from radicle and proximal parts of cotyledon after the third subculture, produced somatic embryos in OMc medium with no hormone, in dark and 16 h light/8 h dark treatments (Table 1, Figs. 1 and 2). Culturing in mediums with IBA and NAA, with subsequent subculturing in the same medium without omission of auxin, prevented somatic embryogenesis. Distal parts of cotyledons had no embryogenesis in any of the treatments.

**Table 1.** Mean number of somatic embryogenesis from calli of different parts of two cultivars' mature embryo, in different photoperiods

Cultivar	Explant-Photoperiod	Mean	DMRT*	
			0.05	0.01
<i>Mission</i>	Radicle-dark	1.222	A	A
	Radicle-light	0.875	A	AB
	Proximal-dark	0.375	B	BC
	Proximal-light	0.182	B	C
<i>Kroneiki</i>	Radicle-dark	0.889	B	AB
	Radicle-light	0.333	B	BC
	Proximal-dark	0.286	B	C
	Proximal-light	0.182	B	C

\*Duncan Multiple Range Test

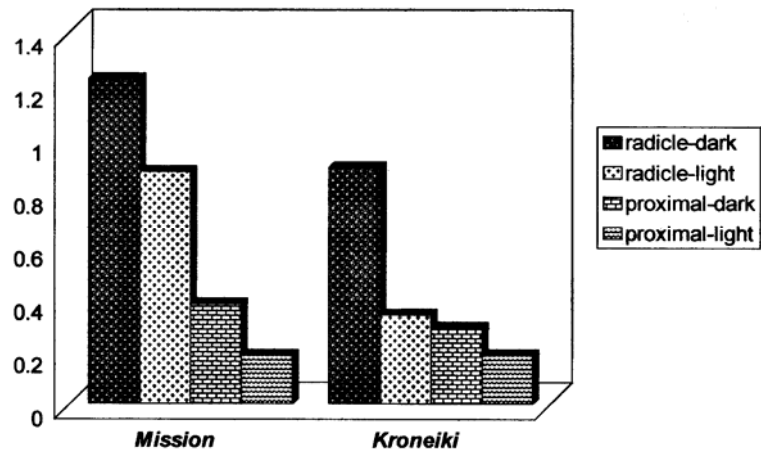


Figure 1. Mean number of somatic embryogenesis from calli of different parts of two cultivars' mature embryo, in light or dark.

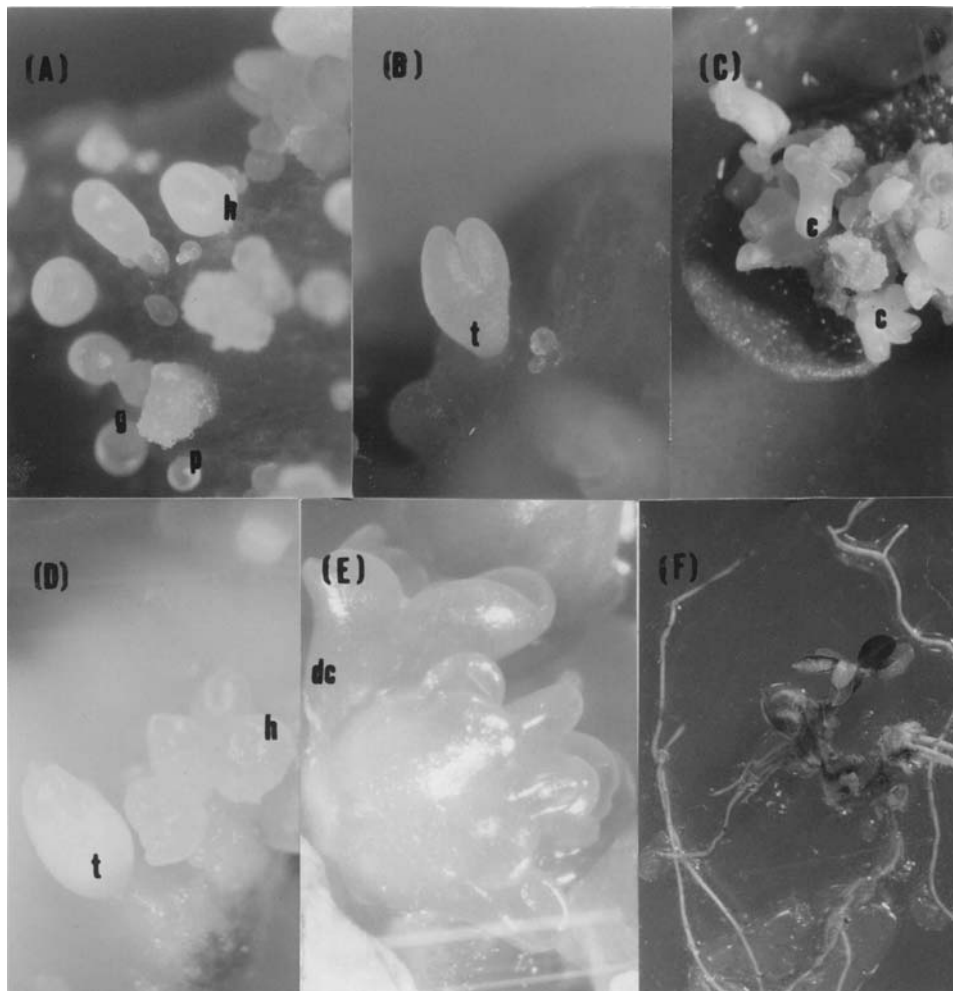
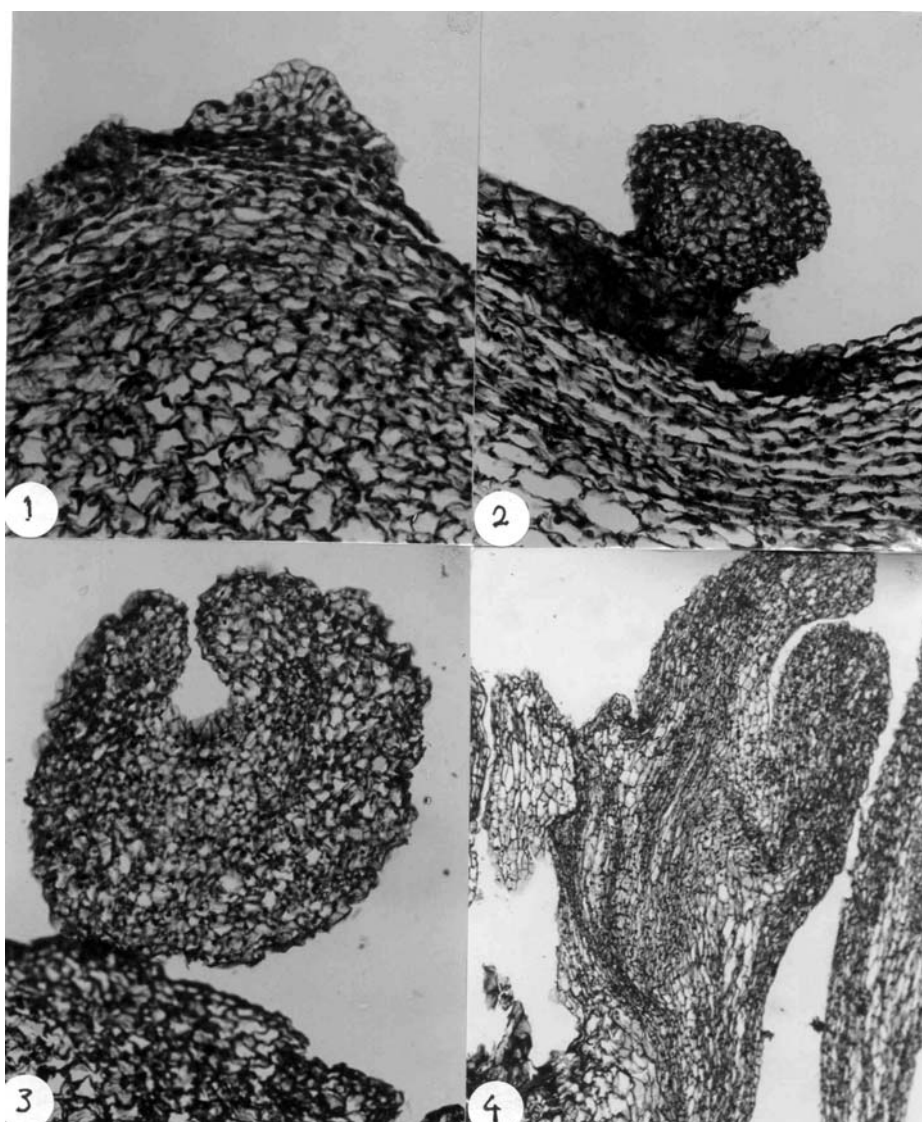


Figure 2. Somatic embryo and normal plantlet production from calli of radicle; (A, B, C): Preglobular (p), globular (g), heart (h), torpedo (t) and cotyledonary (c) stages (cv. *Kroneiki*); (D): Heart (h) and torpedo (t) stages (cv. *Mission*); (E): An embryo with developed cotyledon (dc) and some embryo which coupled from embryo axis (cv. *Mission*); (F): Normal plantlet.



**Figure 3.** Sectioning from embryogenic tissue and developing somatic embryo on the epidermal cells. (1) Embryo development from epidermal and subepidermal cells resulting from superficial anticlinal and basal periclinal division (X594). (2) Embryo at globular stage. The cells are almost in the same size and indicate the same staining and division position (X594). (3) Embryo at late heart stage (X594). (4) Longitudinal section of mature embryo (X394.04).

**(B) *Kroneiki* cultivar.** 21 day-old calli obtained from radicle and proximal parts of cotyledon in OMc medium without any hormones in different photoperiods after the second subculture produced somatic embryos. There was not any embryo production in distal part of cotyledon (Table 1, Fig. 1). The results of analysis of variance indicated that differences between means of somatic embryogenesis in calli of different parts, in different photoperiods and in different cultivars were significant. The differences between samples (calli of radicle and proximal part of cotyledon) in both cultivars were significant (Table 2).

In general, the cellular mass obtained from both *Mission* and *Kroneiki* cultivars, produced some embryo types, consisting of (1) Single embryo (Fig. 2); (2) Double or multiple embryos that coupled from the axis to each other (Figs. 2E). Embryos with developed cotyledons (Fig. 2E). The first type of embryos always produced normal plantlets and the third type produced plantlets that had developed and folded cotyledons.

Histological observation indicated that somatic embryos were produced from epidermal or subepidermal cells (Fig. 3).

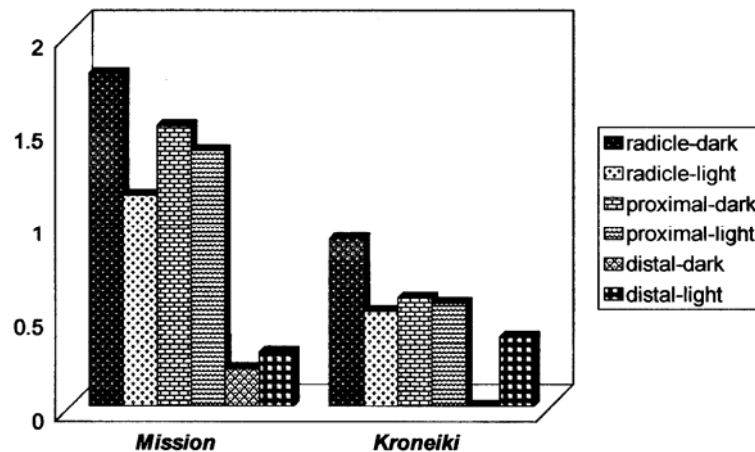


Figure 4. Mean number of rhizogenesis from calli of different parts of mature embryos of the two cultivars, in different photoperiods.

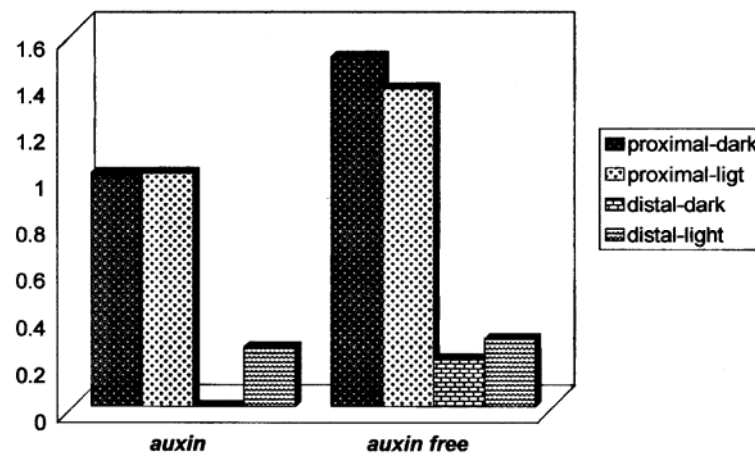


Figure 5. Mean number of rhizogenesis from calli of different parts of mature embryos (cv. *Mission*), in light or dark, with or without auxin in the subculture medium.

### Rhizogenesis

21 day-old calli obtained from different parts of mature embryos of both cultivars in subculture mediums with or without auxin, in different photoperiods, produced roots (Table 3 and Figs. 4 and 5).

Analysis of variance indicated that in both cultivars, difference between rhizogenesis in different parts were significant (Table 4).

### Discussion

Embryogenesis and rhizogenesis of calli obtained from different parts of mature embryos of *Mission* and *Kroneiki* cultivars indicated that (a) the potentiality of embryogenesis and rhizogenesis decreased gradually from the radicle to the distal parts of the embryo; (b) embryogenesis and rhizogenesis in the two cultivars

were different.

The existence of a decreasing gradient of regeneration potentiality from apex to proximal of mature olive embryos [5] and an increased rhizogenesis potentiality of calli obtained from radicle and proximal to distal part of cotyledon [14] and more somatic embryogenesis and rhizogenesis potentiality from radicle to cotyledon parts of mature embryos [12] have been reported. The present results obtained from *Mission* and *Kroneiki* cultivars are comparable with these reports and complete them considering the effects of light conditions.

The fate of cells in tissues and their potentiality to differentiation into other tissues are different. This potential is induced by external conditions such as the kind of medium, growth regulators, photoperiods and etc. [1].

**Table 2.** Analysis of variance of somatic embryogenesis from calli of different parts of mature embryos (Factor A=cultivars, Factor B=explants, Factor C=Photoperiods)

Source	Degree of freedom	Sum of squares	Mean square	F value	F probability
Factor A	1	0.207	0.207	5.3461	0.0231
Factor B	1	1.162	1.162	30.032	0.0000
AB	1	0.134	0.134	3.4660	0.0660
Factor C	1	0.321	0.321	8.2945	0.0050
AC	1	0.006	0.006	0.1680	
BC	1	0.057	0.057	1.4855	0.2262
ABC	1	0.029	0.029	0.7418	
Error	88	3.405	0.039		

**Table 3.** Mean number of rhizogenesis from calli of different parts of two cultivars' mature embryo, in different photoperiods

Cultivar	Explant-Photoperiod	Mean	DMRT*	
			0.05	0.01
<i>Mission</i>	Radicle-dark	1.778	A	A
	Radicle-light	1.125	B	ABC
	Proximal-dark	1.5	AB	AB
	Proximal-light	1.364	AB	AB
	Distal-dark	0.2	DE	DE
	Distal-light	0.286	DE	DE
<i>Kroneiki</i>	Radicle-dark	0.889	BC	BCD
	Radicle-light	0.500	CD	CDE
	Proximal-dark	0.571	CD	CDE
	Proximal-light	0.545	CD	CDE
	Distal-dark	0.00	E	E
	Distal-light	0.364	DE	DE

\*Duncan Multiple Range Test

Photoperiod has an important role in embryogenesis. It was observed that calli of different parts of embryos had a greater potential of embryogenesis in dark than in light. The role of photoperiod in a stage of embryogenesis from immature embryos has been studied [15,18]. It seems that gene expression during embryogenesis is hierarchical and the earliest events involving gene products, presumably act to limit the extent of cell division in response to chemical and/or biophysical factors available to those cells. Clearly, mistakes in the regulation of such early events can disrupt the normal course of the embryogenic pattern [10].

**Table 4.** Analysis of variance of rhizogenesis from calli of different parts of mature embryos (Factor A=cultivars, Factor B=explants, Factor C=Photoperiods)

Source	Degree of freedom	Sum of squares	Mean square	F value	F probability
Factor A	1	1.305	1.305	26.3054	0.0000
Factor B	2	2.835	1.418	28.5732	0.0000
AB	2	0.497	0.249	5.0106	0.0081
Factor C	1	0.051	0.051	1.0243	0.3135
AC	1	0.043	0.043	0.8641	
BC	2	0.424	0.212	4.2706	0.0162
ABC	2	0.009	0.005	0.0939	
Error	120	5.954	0.050		

The existence of IBA and 2-ip in the culture medium and their omission in subculture media is necessary for somatic embryogenesis. Auxin and other growth regulator have effect on differentiation. Auxins are considered as the best inducers of embryogenic cells in several species [7,13]. They have the ability to mediate the transition of somatic cells into embryonic cells.

It is plausible, but unproven, that exogenously applied plant growth regulators directly modify cell polarity [7]. However, it is not clear whether cells in transition between the somatic and embryogenic states are under the influence of exogenous growth regulators or whether they receive specific signals from neighboring cells [13]. Perhaps, there are not any specific signals in the distal part of the cotyledon and the cells of this part do not have the ability of embryogenesis in every condition.

The ability of embryogenic calli for continuous production of somatic embryos without loss of morphogenetic capacity is important. This technique may be used for mass propagation and also for recovery of plants from transformed cells by *Agrobacterium*. Since the epidermal and subepidermal cells bear somatic embryos, these cells should transform easier with *Agrobacterium*. However, these results and other reports [4-6,9-11] indicated that *in vitro* embryogenesis could be possible for some cultivars and for faster multiplication and production of new lines of olive with genetic engineering methods, mutagenesis and somaclonal variation.

## References

1. Ebrahimzadeh, H. Plant Physiology, Vol. II (4<sup>th</sup> Ed.), Tehran University Press, Tehran, p. 689, (1987).
2. Bajaj, Y. P. S. Olive (*Olea europea* L.) Biotechnology in agriculture and forestry. Vol. I; Trees. Springer-Verlag, Berlin, (1986).

3. Bao, Z. H., May, F., Liu, J. F., Wang, K. J., Zhang, P. F., Ni, D. X. and Yang, W. Q. Induction of plantlet from the hypocotyl of *Olea europaea* L. *in vitro*, *Acta Bot. Sin.*, **22**, 96-97, (1980).
4. Canas, L. A., Carramolino, L. and Vieents, M. Vegetative propagation of olive tree from *in vitro* cultured embryos. *Plant Science*, **50**, 85-90, (1987).
5. Canas, L. A. and Benbadis, A. *In vitro* plant regeneration from cotyledon fragments of the olive tree (*Olea europaea* L.), *Ibid.*, **54**, 65-74, (1988).
6. Dadashian, A. The study of lipid quantity and quality in olive plants and explants. M.S. Thesis, Faculty of Science, University of Tehran, (1996).
7. Jong, A. J., Schmidt, D. L. and Vries, S. C. Early events in higher-plant embryogenesis, *Plant Molecular Biology*, **22**, 367-77, (1993).
8. Leitao, L., Duque, A. S. and Fevereço, P. *In vitro* culture of Portuguese varieties of *Olea europaea* L. Some aims and results. *Olivae*, **66**, 54-55, (1997).
9. Leva, A., Muleo, R. and Peturccelli, R. Long-term somatic embryogenesis from immature olive cotyledons. *Journal of Horticultural Science*, **70**(3), 417-21, (1995).
10. Lindsey, K. and Topping, J. F. Embryogenesis: A question of pattern, *Journal of Experimental Botany*, **44**(254), 359-74, (1993).
11. Mencuccini, M. and Rugini, E. *In vitro* shoot regeneration from olive cultivar tissues, *Plant Cell, Tissue and Organ Culture*, **32**, 283-88, (1993).
12. Mitrakos, K., Alexaki, A. and Papadimitriou, P. Dependence of olive morphogenesis on callus origin and age. *Plant Physiology*, **139**, 269-73, (1991).
13. Mordhorst, A. P., Toonen, M. A. J. and Vries, S. C. Plant embryogenesis, *Critical Reviews in Plant Sciences*, **16**(6), 535-76, (1997).
14. Orinos, T. and Mitrakos, K. Rhizogenesis and somatic embryogenesis in calli from wild olive (*Olea europaea* var. *sylvestris*) (Miller). *Plant Cell Tissue and Organ Culture*, **27**, 183-87, (1991).
15. Payvandi, M. The potential of neoformant and transformant embryo and plantlet production in relation to quality and quantity of their lipids. Ph.D. Thesis, Faculty of Science, Science and Research Unit, Islamic Azad University, (1999).
16. Purohit, S. S. Agricultural Biotechnology, (1<sup>st</sup> Ed.), Agro Botanica Publishers & Distributors, New Delhi, India, (1999).
17. Rugini, E. *In vitro* propagation of some olive (*Olea europaea* L.) cultivars with different root ability, and medium development using analytical data from developing shoots and embryos, *Scientia Horticulturae*, **24**, 123-34, (1984).
18. Rugini, E. Somatic embryogenesis and plant regeneration in olive (*Olea europaea* L.), *Plant Cell Tissue and Organ Culture*, **14**, 207-14, (1988).
19. Rugini, E. and Caicato, G. Somatic embryogenesis and plant recovery from mature tissues of olive cultivars (*Olea europaea* L.) "Canino" and "Moralio", *Plant Cell Reports*, **14**, 257-60, (1995).
20. Rugini, E. and Lavee, S. Olive, Biotechnology of Perennial Fruit Crop, Hommerschleg, F. A. and Litz, R. E. (Eds.), International Wolling Oxon, U.K., pp. 371-382, (1993).