Australian Journal of Agricultural Engineering AJAE
AJAE 1(4):126-131 (2010) ISSN:1836-9448

Effect of IAA and 2,4-D on somatic embryogenesis and pigments synthesis of carrot root secondary phloem

S.J. Mousavizadeh[1], K. Mashayekhi[1], V. Akbarpour*[2], H. Kalati[1] and Y. Ghasemi[1]

[1] Department of Horticultural Science, Gorgan University of Agricultural Sciences & Natural Resources, Iran
[2] Department of Horticultural Science, Sari University of Agricultural Sciences & Natural Resources, Iran

***Corresponding author: v_akbarpour60@yahoo.com; vahidakbarpour@gmail.com**

Abstract

Plants secondary metabolites are widely used in medicinal and cosmetic industries. These compounds can be produced *by in vitro* culture of plants. The production of secondary metabolites and somatic embryogenesis *in vitro* are affected by different factors such as the type and concentration of plant growth regulators. In present study, the effect of 2,4-dichlorophenoxyacetic acid (2,4-D) and Indol-3-Acetic Acid (IAA) on anthocyanin, carotenoid, and chlorophylls synthesis as well as somatic embryogenesis of carrot root secondary phloem in liquid and solid media were investigated. Induction of somatic embryogenesis of carrot root secondary phloem was carried out in different concentrations of 2,4-D (0, 2.26, 4.52 and 9.04 µmol) in B5 liquid medium and IAA (0, 2, 5 and 9 µmol) in NL liquid medium. The experiment was conducted in a factorial completely randomized design with four replications. Obtained results showed that liquid medium treatments supplemented with 2,4-D had higher amount of anthocyanin (10.15 µmol/g.f.w) than those supplemented with IAA ($p<0.01$). While the highest amounts of chlorophyll a, b and ab observed in solid medium supplemented with IAA. Also solid medium supplemented with IAA had more carotenoid than liquid medium supplemented with IAA ($p<0.05$). The highest number of somatic embryos produced in media supplemented with 2,4-D ($p<0.01$). In media supplemented with IAA, without proceeding to globular and heart-shape, torpedo-shape embryos and plantlets were formed The highest number of globular-shape and heart-shape embryos formed in media supplemented with 9.04 and 2.26 µmol of 2,4-D, respectively ($p<0.01$).

Keywords: Carrot secondary phloem, Anthocyanin, Carotenoid, Chlorophyll, Somatic embryogenesis.

Introduction

Totipotency is the ability of a single cell to produce different tissues, organs and an organism. Tissue culture is established based on totipotency and may offer manifold benefits in genetic manipulation, clonal propagation of plants and somatic embryogenesis (Mousavizadeh, 2009). The availability of somatic embryogenesis provides excellent experimental material for fundamental studies in embryogenesis (Abdin and Ilah, 2007). Such information can be applied for all plant species (Mashayekhi, 2007; George *et al.,* 2008). Another application of plant tissue culture is the production of secondary metabolites such as anthocyanins and carotenoids (Mashayekhi, 2001). Anthocyanin is a natural plant pigment and a useful secondary metabolite with different pharmacological applications (Edahiro *et al.,* 2005). Epidemiological studies have shown that anthocyanin presence is negatively associated with the morbidity and mortality of cardio and cerebro-vascular diseases and certain types of cancers (Johnsen *et al.,* 2003) Presence of sugar is necessary for anthocyanin synthesis. Anthocyanin synthesis is highly depended on genetic talent of plants. It has been reported that anthocyanin synthesis is correlated with components which increase sugar content of a plant tissue (Hapkins, 1999). Ozeki and Komamine (1981) investigated the anthocyanin synthesis through somatic embryogenesis. Mashayekhi (2001) reported that more evolution in carrot somatic embryos resulted in higher anthocyanin synthesis rate. Edahiro et *al.,* (2005) also reported that L-Phenylalanine is an initial compound of anthocyanin biosynthesis in the phenylpropanoid and flavonoid metabolic pathways of plant cells. Carotenoid synthesis is possible via *in vitro* culture and its production highly depends on plant genotypes and environmental conditions (Meskin et *al.,* 2008). Chlorophyll, the main pigment of plant, synthesis is affected by plant genetic, and different environmental factors such as light, oxygen, carbohydrates, and temperature (Hapkins, 1999). Bercetche *et al.,* (1995) studied the effect of light, sucrose, kinetin and IAA on plastid explants evolution of carrot root secondary phloem. Moreover, Mashayekhi (2001) reported that a positive correlation exists between chlorophyll a, b synthesis and anthocyanin and carotenoid concentrations in plants. However, anthocyanin, carotenoid and chlorophyll synthesis are variable in culture medium. Therefore, further improvement of anthocyanin, carotenoid and chlorophyll accumulation is expected to be achived by variation in concentration of plant growth regulators such as IAA and 2,4-D in different media (solid and liquid) of plant *in vitro* culture. In present studies, somatic embryogenesis and the production of plant pigments of carrot root secondary phloem were investigated in liquid and solid NL and B5 media supplemented by different concentrations of IAA and 2-4,D.

Materials and methods

Plant material, root secondary phloem of carrot (*Daucus carota* L.) cv. Nantes was applied.

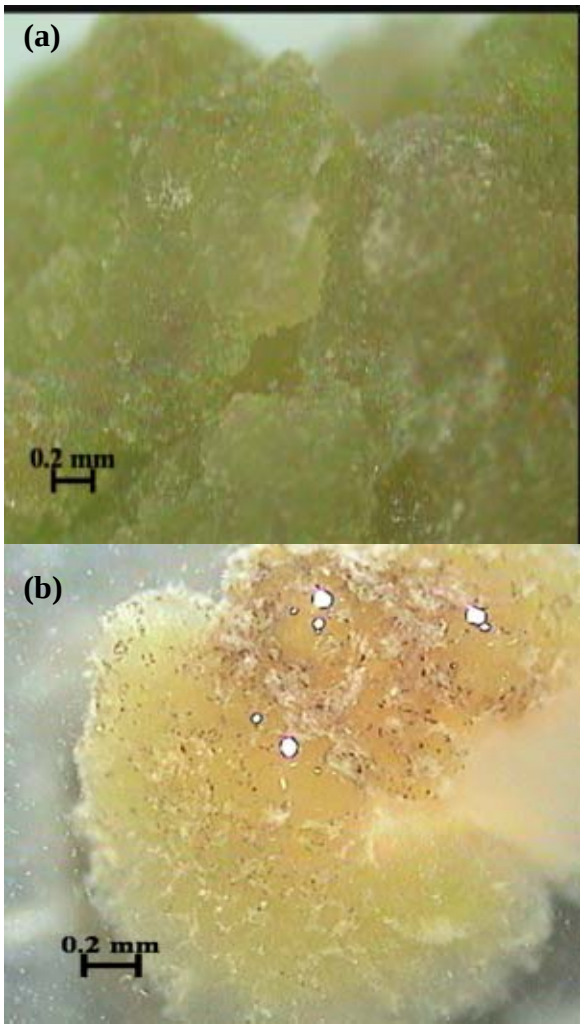

Fig 1. Carrot root secondary phloem explants, after 30 days of culture in liquid NL medium. (a) Increasing of callus chlorophyll in medium supplemented with IAA (5 µM). (b) Increasing of callus anthocyanin in medium supplemented with 2,4-D (4.52 µM).

Surface sterilization

Secondary phloem explants were washed by detergent and under running tap water, then surface sterilization by 70% ethanol for 30 second, and followed by 3% (v/v) Na-Hypochlorite solution for 30 min. One drop of Tween 80 was added to Na-Hypochlorite solution to lower the surface tension. Explants were washed five times with sterile distilled water.

Media and culture condition

The vessels used for the liquid and solid cultures were T-tubes with a capacity of 25 ml and jam jar with a capacity of 30 ml basal nutrient medium, respectively. Solid medium contained 0.08% agar. The pH was adjusted to 5.7 ± 0.1 before autoclaving at 121°C for 15 min. The sterilized explants were cultured on NL basal medium, containing 3% (w/v) sucrose and supplemented with 5 µmol IAA and 4.52 µmol 2,4-D. Carrot root secondary phloem were dissect in 2 mm^2 dimension and were cultured on media supplemented with IAA and 2, 4-D. Five and eight explants of secondary phloem placed on liquid and solid media, respectively. Media incubated at 28 ± 1°C and constant illumination at 2000 Lux. Callus fresh and dry weight, callus moisture percentage, carotenoid and chlorophyll a, b and ab (Arnon, 1956) and anthocyanin contents were measured according to Wanger, (1979) starting 30 days after culture. The amount of anthocyanin, carotenoid and chlorophylls were meseared in the liquid and solid NL media according to Neumann, (1966) method.

Induction of somatic embryos

The ability of somatic embryogenesis of carrot root secondary phloem was investigated by using different concentrations of 2,4-D (0, 2.26, 4.52 and 9.04 µmol) in B5 (Gamborg *et al.*, 1968) and IAA (0, 2, 5 and 9 µmol) in NL liquid medium. Five pieces of secondary phloem explants placed on each container and were incubated at 28 ± 1°C and constant illumination at 2000 Lux. The T-tubes hung on the Auxophyton Steward apparatus, with a rotation of 1.9 rpm. After 3 weeks, the induced organs for the production of somatic embryo, were washed with a hormone free basal medium in three stages for 5, 10 and 15 min and then transferred to a hormone free basal medium. Embryo counting was begun 6 weeks after realization when they started to produced globular, heart, and torpedo shape embryoes which turned to small plantlets. A light microscope (Model; Striyo, Sunny. Monitoring, Sony) was used to examine the specimen for the documentation.

Statistical analysis

Obtained results of physiological tests of root secondary phloem of carrot were normalized by root square ($\sqrt{X}$). Then data were analyzed statistically in a completely randomized design in factorial with two levels of culture media (solid and liquid) and plant growth regulators (2,4-D and IAA) in eight replications. The data of carrot root secondary phloem somatic embryogenesis tests were normalized by root square $\sqrt{X + 0.5}$. Data were analyzed statistically (ANOVA) using analysis of variance and differences among the means were determined for significance at $P<0.05$ using Least Significant Difference test and the system program SAS software (2001).

Results and discussions

Calli growth and secondary metabolites synthesis of root secondary phloem

Considerable variations were observed in callus fresh and dry weight, callus moisture content, chlorophyll b and carotenoid for interaction of culture medium and hormone type (Table 1). Results showed that there was statistically a significant difference between two applied plant growth regulators for anthocyanin contents of explants ($p<0.01$), and a significant difference was observed between two applied media for chlorophyll a and chlorophyll ab (Table 1). As shown in table 2, the highest callus fresh weight and callus moisture content were obtained in solid medium supplemented with 2,4-D; while both solid medium supplemented with 2,4-D and liquid medium supplemented with IAA produced the highest callus dry weights ($p<0.01$). Therefore, type of growth regulator was more effective than medium type on growth of carrot callus. Pierik (1997) believed that better gas exchange in liquid medium than solid medium would reduce callus dehydration. Our results in this study indicate that solid medium supplemented with IAA resulted in higher amounts of chlorophyll a, b and ab than other treatments (Fig. 1). Chlorophyll a content in solid medium containing IAA

Table 1. ANOVA of Carrot root secondary phloem culture.

S.O.V	Df	Callus Fresh Weight (mg)	Callus Dry Weight (mg)	Callus Moisture (%)	Chlorophyll a (mg/g.F.W)	Chlorophyll b (mg/g.F.W)	Chlorophyll ab (mg/g.F.W)	Carotinoid (mg/g.F.W)	Anthocyanin (µmol/g.F.W)
Medium	1	0.064**	0.0023^{ns}	6.54**	0.006*	0.04**	0.035**	0.0002^{ns}	1.07^{ns}
Hormone	1	0.026*	0.002^{ns}	3.55*	0.000001^{ns}	0.00003^{ns}	0.0001^{ns}	0.0005^{ns}	9.55**
Medium × Hormone	1	0.43**	0.068**	34.59**	0.0013^{ns}	0.008*	0.007^{ns}	0.0031*	1.79^{ns}
Error	28	0.006	0.002	0.47	0.001	0.001	0.0024	0.00039	0.72

** ($p<0.01$), * ($p<0.05$), ^{ns} ($p>0.05$)

Table 2. Comparison of carrot root secondary phloem culture traits.

Medium	Hormone	Callus Fresh Weight (mg)	Callus Dry Weight (mg)	Callus Moisture (%)	Chlorophyll a (mg/g.F.W)	Chlorophyll b (mg/g.F.W)	Chlorophyll ab (mg/g.F.W)	Carotenoid (mg/g.F.W)	Anthocyanin (µmol/g.F.W)
Solid	2,4-D	0.421^{a}	0.037^{a}	38.45^{a}	0.021^{ab}	0.024^{b}	0.045^{ab}	0.02^{b}	3.9^{ab}
	IAA	0.128^{c}	0.013^{b}	11.5^{c}	0.025^{a}	0.04^{a}	0.065^{a}	0.032^{a}	1.93^{b}
Liquid	2,4-D	0.104^{c}	0.006^{b}	9.72^{c}	0.014^{ab}	0.009^{c}	0.023^{bc}	0.026^{ab}	10.15^{a}
	IAA	0.259^{b}	0.041^{a}	21.87^{b}	0.011^{bc}	0.006^{c}	0.017^{c}	0.021^{b}	1.59^{b}
LSD 5%		0.057	0.014	4.731	0.0096	0.0092	0.0186	0.0063	6.21

Means followed by the same letter are not significantly different at 5% by LSD.

Table 3. Correlation among carrot root secondary phloem culture traits.

Traits	Callus Fresh Weight (mg)	Callus Dry Weight (mg)	Callus Moisture (%)	Chlorophyll a (mg/g.F.W)	Chlorophyll b (mg/g.F.W)	Chlorophyll ab (mg/g.F.W)	Carotinoid (mg/g.F.W)	Anthocyanin (µmol/g.F.W)
Callus Fresh Weight (mg)	1							
Callus Dry Weight (mg)	0.87**	1						
Callus Moisture (%)	0.99**	0.81**	1					
Chlorophyll a (mg/g.F.W)	-0.11^{ns}	0.01^{ns}	-0.14^{ns}	1				
Chlorophyll b (mg/g.F.W)	-0.10^{ns}	-0.06^{ns}	-0.11^{ns}	0.89**	1			
Chlorophyll ab (mg/g.F.W)	-0.12^{ns}	-0.04^{ns}	-0.14^{ns}	0.96**	0.97**	1		
Carotinoid (mg/g.F.W)	-0.54*	-0.47^{ns}	-0.51^{ns}	0.07^{ns}	0.28^{ns}	0.21^{ns}	1	
Anthocyanin (µmol/g.F.W)	-0.09^{ns}	-0.25^{ns}	-0.06^{ns}	-0.07^{ns}	-0.04^{ns}	-0.06^{ns}	0.02^{ns}	1

** ($p<0.01$), * ($p<0.05$), ^{ns} ($p>0.05$)

Table 4. ANOVA of carrot root secondary phloem somatic embryogenesis.

S.O.V	df	Globular	Heart	Torpedo	Plantlet	Neomoroph	Total embryo
Hormone	1	24.71**	5.96**	0.33*	5.42**	12.2**	63.45**
Concentration	3	12.55**	1.11**	0.05^{ns}	1.91**	1.45**	19.41**
Concentration × Hormone	3	3.45**	1.11**	0.058^{ns}	1.9**	1.45**	8.07**
Error	24	0.08	0.19	0.053	0.07	0.2	0.07

** ($p<0.01$), * ($p<0.05$), ^{ns} ($p>0.05$)

showed a significant difference with its amounts in liquid medium supplemented with IAA ($p<0.05$); while no significant difference observed in other treatments for chlorophyll a. Our findings are in concordance with Bercetche *et al.*, (1995) results who showed that presence of plastids in cells increased by adding IAA to medium of carrot root secondary phloem containing sucrose (0.02). Higher amounts of carotenoid were observed in solid medium containing IAA than liquid medium supplemented with IAA ($p<0.05$). Therefore, solid medium contained more chlorophyll and carotenoid than liquid medium. Based on Pierik's suggestion (1997), solid culture medium results in quick callus differentiation. Results of correlation coefficient showed that a significantly negative correlation exists between callus wet weight and carotenoid content (Table 3). In medium supplemented with 2,4-D, higher amounts of anthocyanin obtained in compare with of the one supplemented with IAA. Also liquid medium supplemented with 2,4-D showed the highest amounts of anthocyanin of all treatments (Table 2). This finding is in accordance with Mashayekhi (2001), who reported that the increase of 2,4-D concentration during somatic embryo initiation of carrot petiole caused an increase in the amounts of carotenoid and anthocyanin. Edahiro et *al.*, (2005) also applied 2,4-D for determination of anthocyanin accumulation in cell culture of strawberry. It seems that 2,4-D causes an stress in plant an lead to increase of anthocyanin synthesis (Shariatpanahi *et al.*, 2006). Jimenez and Bangerth (2001) also reported that medium supplemented with 2,4-D for carrot callus initiation showed an increase of abscisic acid which results in higher anthocyanin synthesis. In addition, Rahimi (2008) reported that salicylic acid like 2,4-D increased the anthocyanin content of *in vitro* plants significantly. Plants under stress condition have a defensive antioxidant system which omits free radicals of oxygen and anthocyanins are one of the most important antioxidant compounds. Besides, anthocyanins act as light protective pigments in plant cells during stress period (Meskin et al., 2008). As demonstrated in figure 1-b, red calli represents high levels of anthocyanin in liquid medium supplemented with 4.54 µM 2,4-D. For the same treatment lower amounts of chlorophyll a, b and ab observed in comparison to other treatments. Based on correlation coefficient results, no significant correlation observed between anthocyanin and the mentioned types of chlorophyll (Table 3). Our findings are in accordance with Hapkins (1999) who stated that increase of anthocyanin doesn't interfere with photosynthesis of mesophyll cells.

Somatic embryogenesis of root secondary phloem

As shown in table 4, a statistically significant difference exists between interaction of plant growth regulators and their concentrations for globular-shaped heart-shaped, plantlet-shaped neomoroph-shaped and total embryos ($p< 0.01$); while no significant difference observed for torpedo-shaped embryo ($p>0.05$). Based on mean comparison, the most number of embryos observed in media supplemented with 2,4-D. For the media supplemented with IAA, no heart-shaped, torpedo-shaped, plantlet-shaped, and neomoroph-shaped embryos were formed and all embryos remained in

Fig 2. Incomplete embryo with interconnected cotyledons (Neomoroph) from carrot root secondary phloem explants in NL liquid medium supplemented with 2,4-D (4.52 µM), 6 weeks after realization.

globular-shaped stage. The highest number of globular-shaped (4.75), plantlet-shaped (2.63), and total embryos (6.28) were counted in medium supplemented with 9.04 µM 2,4-D ($p<0.01$); while the highest number of heart-shaped embryos were recorded in medium supplemented with 2.26 µM 2,4-D ($p<0.01$). Mashayekhi (2001) expressed that more somatic embryos produced in carrot petiole explants which possess more meristemic tissue than secondary phloem. Therefore, carrot secondary phloem is more differentiated than petiole tissue which produces less somatic embryos. But the most important difference between plant tissues in embryonic and non-embryonic cultures is their different endogenous hormone levels. Jimenez and Bangerth (2001) reported a positive correlation between the ability of response to exogenous 2,4-D initiation and endogenous IAA levels of explants. On the other hand, Shariatpanahi *et al.*, 2006 stated that effect of 2,4-D on endogenous IAA metabolism plays an important role in somatic embryogenesis. Several researchers have applied 2,4-D in their tests for its long stability and maintenance in culture medium and suggested it as the most effective hormone in somatic embryogenesis initiation. In addition, embryonic cells are easily formed on media supplemented with 2,4-D for carrot explants (Jimenez and Bangerth (2001). IAA can be used only for the induction of embryogenesis. Similarly, Mashayekhi (2001) suggested that since IAA is an unstable auxin and decomposed after 4 to 6 days exposure to light, embryonic cells on media supplemented with IAA don't need to be subcultured in a new growth regulators-free medium. Therefore, this is a benefit of IAA application for somatic embryogenesis, which results in the reduction of pollution risk in the subcultured medium.

Table 5. ANOVA of carrot root secondary phloem somatic embryogenesis.

Hormone	Concentration (µmol)	Globular	Heart	Torpedo	Plantlet	Neomoroph	Total embryo
2,4-D	0	0.7^{f}	0.7^{c}	0.7^{a}	0.7^{c}	0.7^{b}	0.7^{f}
	2.26	3.22^{c}	2.49^{a}	1.05^{a}	0.7^{c}	2.18^{a}	4.7^{c}
	4.52	4.13^{b}	1.35^{b}	1.05^{a}	2.07^{d}	2.22^{a}	5.37^{b}
	9.04	4.75^{a}	1.74^{b}	0.83^{a}	2.63^{a}	2.18^{a}	6.28^{a}
IAA	0	0.7^{f}	0.7^{c}	0.7^{a}	0.7^{c}	0.7^{b}	0.7^{f}
	3	0.7^{f}	0.7^{c}	0.7^{a}	0.7^{c}	0.7^{b}	0.7^{f}
	6	2.59^{d}	0.7^{c}	0.7^{a}	0.7^{c}	0.7^{b}	2.59^{d}
	9	1.78^{e}	0.7^{c}	0.7^{a}	0.7^{c}	0.7^{b}	1.78^{e}
LSD 5%		0.41	0.63	0.36	0.41	0.63	0.38

Means followed by the same letter are not significantly different at 5% by LSD.

Neomoroph embryo

Mostly in suspension cell culture an structure is observed which is similar to somatic embryogenesis but formed abnormally. These are in fact somatic embryos evolutes abnormally and considered "neomoroph" embryos. Neomoroph embryos initiated in medium but don't get free in the form of complete somatic embryos (Mashayekhi, 2001). Mousavizadeh, (2009) also reported the presence of neomoroph embryos in somatic embryogenesis of strawberry culture. In the present study, the highest number of neomoroph embryos observed in medium supplemented with 2,4-D (Fig. 2). Mashayekhi (2007) reported that formation of neomoroph embryos occurs in medium supplemented with an strong auxin such as 2,4-D. Based on somatic embryogenesis researchers' suggestion, formation of neomoroph embryos and loss of evolution to a complete plantlet depends highly to endogenous auxin and high levels of abscisic acid in the medium (John *et al.*, 1995, Mashayekhi, 2001). Tran Thi, (2000) reported that abscisic acid concentrations higher than 10^{-7} M act as growth, evolution and differentiation inhibitor, and it results in formation of abnormal somatic embryos. For somatic embryogenesis of pine (*Pinus pinaster*), neither evolution of embryos nor achieving to cotyledon stage was attributed to presence of abscisic acid in the medium (Bercetche and Paques. 1995). In our research, for callus induction of carrot root secondary phloem, more anthocyanin observed in media supplemented with 2,4-D than the one supplemented with IAA. As mentioned above, 2,4-D causes stress in plants (Shariatpanahi et al., 2006) and may result in higher anthocyanin production (Meskin et al., 2008). Therefore, 2,4-D application in medium causes higher abscisic acid synthesis and increase anthocyanin concentration which results in neomoroph embryos formation (Jimenez and Bangerth, 2001; Mashayekhi, 2001).

Conclusion

Comparison of solid and liquid media and growth regulators supplement to the nutrient solution is of significant importance to evaluate. Since, *in vitro* secondary metabolites synthesis and somatic embryogenesis are affected by the combination of nutritional material and growth regulators in the medium. In the present study, calli of carrot root secondary phloem revealed the highest amounts of carotenoid, chlorophyll a, b and ab in the media supplemented with IAA. Also the highest anthocyanin content recorded in media supplemented with 2,4-D as compared to the one supplemented with IAA. Media supplemented with 2,4-D caused significant increase in the number of globular, hear, plantlet and neomoroph shaped embryos. Therefore, 2,4-D is thought to play an important role in embryogenesis**.** It seems to be arisen from the fact that 2,4-D cause an increase in callus anthocyanin content and neomoroph shaped embryos formation by inducing stress and abscisic acid levels in culture. Therefore, results of this study demonstrated that increase or alteration of anthocyanin, carotenoid and chlorophyll levels *in vitro* cultures are possible by alteration in composition type of media and growth regulators. However, secondary metabolites production through somatic embryogenesis needs further investigations.

References

Abdin MZ, Ilah A (2007) Plant regeneration through somatic embryogenesis from stem and petiole explants of Indian chicory (*Cichorium intybus* L.). Indian Journal of Biotechnology, 6:250-255.

Arnon DI (1956) Photo synthesis by isolated chloroplast. Biochem & Biphy. 20:440-461.

Bercetche J Paques M (1995) Somatic embryogenesis in maritime pine (*Pinus pinaster*). In Jan, M. S., Gupta, P.K. and R.J. Newton (eds.), Somatic Embryogenesis in woody Plants, Kluwer Academic Publishers. Netherlands, Vol. 3: 221-242.

Edahiro J, Nakamura M., Seki, M. and Fursaki, Sh (2005) Enhanced Accumulation of Anthocyanin in Cultured Strawberry Cells by Repetitive Feeding of L-Phenylalanine into the Medium. Journal of Bioscience and Bioengineering, Vol. 99, 1: 43-47

Gamborg OL, Miller RA, Ojima K (1968) Nutrient requirements of suspension cultures of soybean root cells. Exp. Cell Res. 50:151-158.

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

Hapkins WG (1999) Introductin to plant physiology. Vol 1 and 2, John Wiley and Sons, New York.

Jimenez VM, Bangerth F (2001) Endogenous hormone levels in explants and in embryogenic and non- embryogenic culture of carrot. Physiologia Plantarum. 111: 389-395.

John A, Drake P Selby C (1995) Somatic embryogenesis in Sitka spruce (*Picea sitchensis* (Bong.) Carr.). In: Jan, M.S., Gupta, P.K. and R.J. Newton (eds.), Somatic Embryogenesis in woody Plants, Kluwer Academic Publishers. Netherlands. 3: 125-143.

Johnsen S, Overvad K, Stripp C, Tjonneland A, Husted SE, Sorensen HT (2003) Intake of fruit and vegetables and the risk of ischemic stroke in a cohort of Danish men and women. American Journal of Clinical Nutrition, 78: 57–64.

Mashayekhi K (2001) The protein synthesis spectrum during the induction phase of somatic embryogenesis in carrot (*Daucus carota* L.) cultures and the role of nitrogen forms for embryo development. A thesis of Doctor of Science in Agriculture. Justus Liebig University, Giessen. 95 p.

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

Meskin, M. S., Bidlack, W. R and Randolpha, R. K (2008) Phytochemicals, aging and health. CRC Press. P 228.

Mousavizadeh SJ (2009) The investigation of Strawberry and Carrot petiole explants behavior has been excised in vitro. Gorgan University of Agricultural Science and Natural Resoures, Iran. M.Sc thesis. 108 p.

Neumann KH (1966) Wurzelbildung und Nukleinsauregehalt bei phloem Gewebekulturen der karrotenwurzel auf synthetischen Nahrmedium verschiedener Hormonko-mbina-tionen. Lees Phytohormones ET Organogenese. 38:95-102.

Ozeki Y, Komamine A (1981) Induction of anthocyanin synthesis in relation to embryogenesis in a carrot suspension culture: Correlation of metabolic differentiation with morphological differentiation. Physiol, Plant. 53:570-577.

Pierik RLM (1997) *In vitro* culture of higher plants. Dordrecht, Boston. Kluwer academic publisher. 348 p.

Rahimi A (2008) Effect of Nitrogen, Phosphorus, Potassium, Mangsium, Zinic, Iron, Boron and Molybdenum on growth, yeild and Essential oil in Coriander. *(Coriandrum sativum L.*). Gorgan University of Agricultural Science and Natural Resoures, Iran. M.Sc thesis. 120 p

SAS Institute (2001) SAS/STAT user´s guide. Version 9. SAS Institute, Cary, N.C.

Shariatpanahi ME, Bal U, Heberle-Bors E, Touraev A (2006) Stresses applied for the re-programming of plant microspores towards in vitro embryogenesis. Physiologia Plantarum. 127:519-534.

Tran Thi L (2000) Untersuchungen zur Bedutung der Abscisinsaure (ABA) fur die somatische Embryogenese verschiedener Daucusarten unter Einbeziehung transgener Stamme. Dissertation zur erlangung des Doktorgrades der Agrarwissenschaften der Justus-liebig- Universitat Giessen. In Mashyekhi, K. 2007. Plant Somatic Embryogenesis. Makhtomgholi Fraghi (Sarli) press. 488 p

Wanger, G.J (1979) Content and vacuole\ extra vacuole distribution of neutral sugars, free amino acids and anthocyanins in protoplasts. Plant physiology. 64:88-93.