WFL Publisher
Science and Technology
Meri-Rastilantie 3 B, FI-00980
Helsinki, Finland
e-mail: info@world-food.net

Journal of Food, Agriculture & Environment Vol.8 (2): 325-329. 2010 *www.world-food.net*

Analysis of *in-vitro* explants mineral contents to modify medium mineral composition for enhancing growth of Persian walnut (*Juglans regia* L.)

Elahe Najafian Ashrafi [1], Kourosh Vahdati [2]*, Hassan Ebrahimzadeh [1] and Masoud Mirmasoumi [1]
[1] Department of Plant Sciences, Faculty of Biology, College of Sciences, University of Tehran, Tehran, Iran. [2] Department of Horticulture, College of Abouraihan, University of Tehran, Pakdasht, Tehran, P.O. Box 33955-159, Iran.
**e-mail: kvahdati@ut.ac.ir*

Received 18 January 2010, accepted 11 April 2010.

Abstract

Micropropagation of Persian walnut (*Juglans regia* L.) still needs more study to be used consistently in various cultivars. Due to slow growth and yellow appearance of Persian walnut explants in DKW medium, a trial was conducted to refine the DKW medium composition. The method consists of adding the mineral composition that the walnut explants contain in the period of maximum growth to the DKW medium. The mineral composition of the explants of two walnut cultivars (Sunland and Chandler) were determined by ICP-OES technique. Two modified media (mDKW) were formulated based on the walnut explant mineral contents. Because of inappropriate concentration of nitrogen and calcium, media with two ranges of calcium and nitrogen were formulated; 1) $mDKW_1$ (DKW with 6 modified macronutrient concentrations) and 2) $mDKW_2$ (DKW with 4 modified macronutrient concentrations). Other minerals were formulated based on explant mineral concentrations. The growth rate of explants cultured on mDKW was compared to those cultured on DKW medium. There were no deficiency symptoms in explants in mDKW. Among the cultivars, Chandler had the maximum stem length and auxiliary bud number. Explants grown on $mDKW_2$ produced the maximum stem length and auxiliary bud number. Also the explants grown on $mDKW_2$ showed no deficiency and yellow color leaves after 30 days. The results suggested that micropropagation in a medium containing mineral balance similar to that in explants may hold a hypertonic concentration of minerals for them to move from medium to explant and therefore will obtain better growth and proliferation conditions for explants.

***Key words*:** Proliferation, DKW, element, nutrient, walnut.

Introduction

Chemical composition of the culture medium plays an important role in the success of micropropagation [35]. The deficiency of minerals in plants can cause biochemical, physiological and morphological changes, according to the nutrient and to the level of deficiency [24, 32]. The difficulty of optimizing the culture medium, because of interactions between different medium components, has led to the employment of various tissue culture media [31, 32].

Generally, two strategies are used to define the balance between the different minerals and organic elements for the optimal growth of the plant cells or nutrients for one species by studying the interaction effect of each of them on the others. One of the best known examples is the work of Murashige and Skoog [28] who established an optimal nutrient composition of the culture medium for rapid growth with tobacco callus cultures. Another strategy is adapting the mineral content of the medium according to an elemental analysis of healthy plant tissue [8].

The optimization of mineral composition in the culture medium based on elemental analysis of various plant parts has been frequently reported. The mineral compositions of various plant parts have been used for this purpose, including a combination of *in vitro* grown apical shoots and mature zygotic embryos [34], seeds [16, 31] and whole plants [2, 3, 10, 11, 24, 38].

The potential of tissue culture techniques for the propagation of Persian walnut has been studied by many researchers. Driver and Kuniyuki [6] using Cheng [41], Murashige and Skoog (MS) [28], B_5 [9] and woody plant media (WPM) [17] encountered the problem of gradual culture deterioration. This problem led them to develop a new medium called DKW, optimized especially for the growth of the paradox hybrid walnut.

Although DKW medium was developed for paradox hybrid walnut, it has been proven suitable for a variety of Juglandaceae species, including Persian walnut (*Juglans regia* L.) and is currently the most widely used medium for walnut culture [5, 22, 23]. Despite this medium caused rapid growth of walnut explants, there have been some disorders in some walnut cultivars for propagation and optimum growth. The proliferation of few shoots, low rooting rate and chlorosis of walnut explants are some of the most important problems in DKW medium. In spite of such disorders, only few studies were performed on optimization of this medium [12].

We hypothesized that developing a medium according to the mineral concentration of the explant tissues will be resulted in the best nutritional conditions for growth of explants in the culture medium. Therefore, the aim was to analyse *in-vitro* explants mineral contents to modify medium mineral composition for enhancing growth of Persian walnut.

Materials and Methods

***Plant materials and chemical analysis*:** Two Persian walnut cultivars, namely Sunland and Chandler that had been introduced in *in-vitro* more than 10 years ago and subcultured every 30 days were used as the explant source for chemical analysis. The cultures were maintained at 23±2°C under 7000 lux cool-white fluorescent light for 16-h photoperiod. DKW was used as the culture medium source. The cultures were grown on fresh medium for 30 days since subcultures before the explants were harvested for analysis.

The explants were cleaned by removal of medium material and dried at 70°C for 72 h. The dried samples were then ground to fine powder. For analysis of mineral composition, 250 mg of plant material was dissolved in 3 ml H_2SO_4 (96%) and 10 ml of H_2O_2 (50% w/v), heated and made up to 100 ml with distilled water. Total nitrogen was determined by Kjeldahl method, S by turbidimetric method and P, Mg, Ca and K were analyzed by inductively–coupled plasma atomic emission spectroscopy.

Calculation of the modified medium components: A new medium must hold a hypertonic concentration of macronutrients for the explants to move from medium to explants cells. This condition can be covered by a multiplication factor (α_c) that compares the total leaf macronutrient contents of a particular cultivar (M_1) and total macronutrient content of any widely tried medium (M_r) according to Equation 1 proposed by Terrer and Tomăş [38] :

$$\alpha_c = \frac{\sum \text{reference medium macronutrients}}{\sum \text{explant macronutrients}} = \frac{\sum M_r}{\sum M_1} \qquad \text{(Eq. 1)}$$

By multiplying this factor for each macronutrient found in explants a new specific composition will be obtained.

Measurements and cultural conditions: The explants were maintained in the DKW as a culture medium for 30 d in the following conditions: 25°C and 7000 lux for 16 h per day in the 100-ml glass jars. The explants were harvested for analysis after 4 subcultures. Several quantitative parameters, including stem length, auxiliary bud number, stem width and leaves color of explants were recorded. The revised media contained micronutrients, vitamins and hormones of DKW medium supplemented with 100 mg l^{-1} myo-inositol, 30 g l^{-1} sucrose and 2 g l^{-1} Gelrite (Sigma Co.). The Fe-HEDDA chelate was chosen as the iron source. The pH of the medium was adjusted to 5.5 before autoclaving at 121°C for 20 min. Four shoot tips were transferred to the modified medium and DKW as a control medium. This experiment was conducted with three replications of walnut cultivars.

In vitro rooting of the cultivars was also examined. A two–phase rooting procedure was used according to Vahdati *et al.* [39]. For root induction, shoots of 3-4 cm with fresh cuts at the basal end (to remove any callus) were harvested after 30 days and were placed in the 100-ml glass jars under dark for 7 days on MS medium supplemented with minerals, vitamins of MS medium [28], 30 g l^{-1} sucrose, 15 µM IBA and 2.5 g l^{-1} Gelrite (Sigma Co.). Use of agar and quarter-strength DKW during the induction phase was based on results reported by Navatel and Bourrain [30] and Ripetti *et al.* [33]. The pH of the media was adjusted to 5.5 before autoclaving at 121°C for 20 min. Induction was followed by keeping under light on a mixture of ¼ strength DKW basal medium, 30 g l^{-1} sucrose, 2.5 g l^{-1} Gelrite (Sigma Co.) and vermiculite (1:1.25 v/v) and maintained in the same environment as for multiplication [13]. Rooting percentage was determined after 3 weeks. Four plantlets were used during rooting for each cultivar.

Statistical analysis: Analysis of variance and comparison of means were performed using SAS (SAS Institute Inc., Cary, NC) software. Significant differences among the mean values were compared by Duncan's multiple range test ($P \leq 0.05$).

Results

Table 1 shows the macronutrients found in explants and their corresponding multiplication factors α_c, defined by Equation 2:

$$\alpha_c = \Sigma M_1 / \Sigma M_2 = 25.5 / 13.98 = 179.8 \qquad \text{(Eq. 2).}$$

Macronutrient concentrations for modified medium (g l^{-1}) are also shown in Table 1. The elemental composition of the modified culture medium shows that the most important differences between the modified medium and DKW are an increase in the concentration of N, P, S and a decrease in the concentration of K and Ca (Table 2). The nitrogen concentration of explants was much higher than in DKW medium. Also the calcium concentration of explants and modified medium was 1/3 of that in DKW medium. For this reason, two media with two ranges of calcium and nitrogen concentration as $mDKW_1$ (medium with 6 modified macronutrient concentrations) and $mDKW_2$ (medium with 4 modified macronutrient concentrations) were used. Other minerals were formulated based on explant mineral concentration (Table 3).

Plantlets grown on DKW and mDKWs exhibited differences in growth. Plantlets grown on $mDKW_1$ had smaller leaves, auxiliary bud number and lower stem thickness than on $mDKW_2$ and DKW as a control medium (Fig. 1). However, plantlets grown on $mDKW_2$

Table 1. Explants macronutrient percentages (% dry matter) and multiplication factor (α_c(referred to mean total macronutrients (25.5 mg l^{-1}) of DKW medium in walnut explants ('Sunland'and 'Chandler').

Chemical analysis (% dry matter)							
N	P	K	Mg	Ca	S	ΣM1	$\alpha_c = \Sigma M_1 / \Sigma M_2$
6.65	0.43	3.02	0.51	0.79	2.57	13.98	179.8

Table 2. Macronutrient contents (mg l^{-1}) of modified medium for walnut explants and DKW medium.

Medium	N	P	K	Mg	Ca	S
Modified medium (mDKW)	1195	77.3	544	91.69	142.04	462
DKW medium	783	57	768	72	372	390

Table 3. Mineral composition (mg l^{-1}) of modified media and DKW as a reference medium.

Salt	$mDKW_1$*	$mDKW_2$**	DKW medium
NH_4NO_3* **	**5954**	**1416**	1416
$Ca(NO_3)_2.4H_2O$	**420**	**1968**	1968
$Zn(NO_3)_2.4H_2O$	23.5	23.5	23.5
K_2SO_4	867	867	1559
$MgSO_4.7H_2O$	864	864	740
$MnSO_4.H_2O$	33.5	33.5	33.5
$CuSO_4.5H_2O$	0.25	0.25	0.25
$NiSO_4.6H_2O$	0.005	0.005	0.005
$CaCl_2.2H_2O$	149	149	149
KH_2PO_4	330	330	265
H_3BO_3	4.8	4.8	4.8
$Na_2MoO_4.2H_2O$	0.39	0.39	0.39
Fe-HEDDA	100	100	100

*The medium with 6 modified macronutrient concentrations. ** The medium with 4 modified macronutrient concentrations.*** The differences between two modified media was only at salts as NH_4NO_3 and $Ca(NO_3)_2.4H_2O$ highlighted with bold numbers.

did not exhibit chlorosis in compare to plantlets grown on DKW (Fig. 2).

Among the cultivars, Chandler had the maximum stem length and auxiliary bud number (Table 4). The effect of medium was significant on the stem length and auxiliary bud number ($P \leq 0.05$).

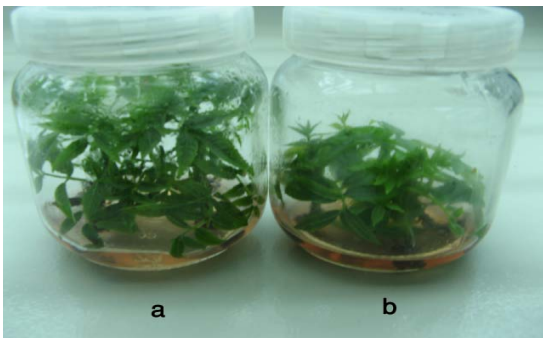

Figure 1. Plantlets of Persian walnut grown *in-vitro* after four subculture cycles on (a) DKW and (b) $mDKW_1$ (medium with 6 modified macronutrient concentrations).

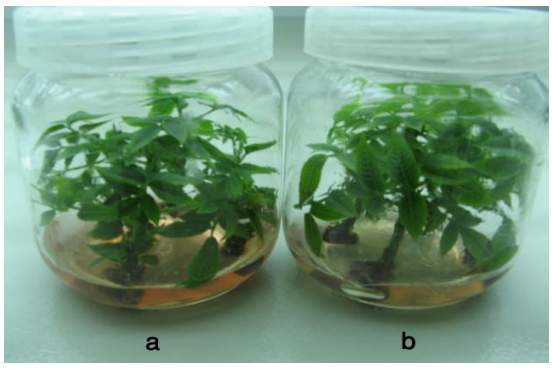

Figure 2. Plantlets of Persian walnut grown *in vitro* after four subculture cycles on (a) DKW and (b) $mDKW_2$ (the medium with 4 modified macronutrient concentrations).

Table 4. Growth rate of Persian walnut cultivars under *in-vitro* condition in the revised media.

Cultivar	Character		
	Auxiliary bud number	Stem length (cm)	Stem width (cm)
Chandler	18.19± 2.12a*	4.67 ± 3.73 a	2.90± 1.75 a
Sunland	17.67± 2.56 a	4.10 ± 0.71 a	2.71 ± 0.33 a

*Means followed by the same letter(s) are not significantly different at $P \leq 0.05$.

Explants grown on $mDKW_2$ produced the maximum stem length and auxiliary bud number and stem thickness among the other media (Table 5). Also the explants grown on $mDKW_2$ showed no deficiency and leaves chlorosis after 30 days. The rooting percentage was decreased in explants cultured on the $mDKW_1$ and $mDKW_2$ comparing to the control. 'Sunland' had the highest root ability in all the media. However, the rooting percentage of $mDKW_2$ was nearly similar to DKW medium (Table 6).

Table 5. Growth rate criteria of Persian walnut under different culture media in 30 days.

Medium	Character		
	Auxiliary bud number	Stem length (cm)	Stem width (cm)
$mDKW_1$***	16.90± 1.64c***	4.66 ± 1.01ab	2.06 ± 2.05a
$mDKW_2$**	19.04± 1.92a	5.83 ± 0.6a	3.01 ± 2.08 a
DKW	18.30 ±2.01ab	3.96 ± 0.46b	2.42 ± 0.44a

*The medium with 6 modified macronutrient concentrations. **The medium with 4 modified macronutrient concentrations. ***Means followed by the same letter(s) are not significantly different at $P \leq 0.05$.

Table 6. Rooting percentage of *in vitro* shoots of two Persian walnut cultivars induced to root on MS medium with 15 µM IBA and 2.5 g l^{-1} in the dark for 1 week and transformed to the development medium in the light for 3 weeks.

Cultivar	Media		
	DKW	$mDKW_1$	$mDKW_2$
Sunland	50a*	27a	42a
Chandler	40b	16ab	35b

*Means followed by the same letter(s) are not significantly different at $P \leq 0.05$.

Discussion

The differences of mineral concentrations between plantlets and the media indicate that there are interactions between minerals in the media or between minerals and the gel matrix [11]. In addition, the differences may be due to different uptake mechanisms possessed by the plant [11].

In $mDKW_1$, the concentration of N is much higher but the Ca content is lower than in DKW medium. On the other hand, in the mentioned medium, the K/Ca ratio is higher than that in DKW. According to Malavolta *et al.*[18], one of the reasons of the Fe deficiency is a high K/Ca ratio. High levels of K cause Fe deficiency but does not affect significantly on Ca [24]. It was supposed to see Fe deficiency in the modified media but the N/P ratio in the media eliminated the Fe deficiency. An imbalance in the N/P ratio may be also involved in the induction of chlorosis. It is proposed that the yellowish color of leaves and deficiency symptoms shown in $mDKW_1$ are caused by imbalance of N/P and K/Ca ratios. In $mDKW_2$, the ratio of these minerals is thought to be optimal for walnut explants.

The most important differences between the modified media and DKW are an increase in the concentrations of N, P, S and a decrease in the concentrations of K and Ca. Although Judd *et al.*[14] believed that the concentrations of N, P, K and B were much higher in tissue cultured plantlets than in greenhouse grown plants, other minerals (Ca and Mg) were found at lower levels *in vitro*. It should be emphasized that analyses of used media showed no exhaustion for any specific mineral. This meant that the differences in growth on the

investigated media were a consequence of differences in relative concentrations of these minerals and therefore differences in relative uptake [3]. In addition, some minerals *in vitro*, in particular N, P and K, were outside the adequate ranges for explants and at levels that could adversely affect the plantlet growth [14].

The higher levels of N, P, Mg and S in walnut explants than those in DKW medium were thought to be caused by subsequent subculturing but the much lower Ca in explants corresponded to low solubility of this element in the medium. In addition, more than one third of the Ca supplied by the culture medium is required by agar or Gelrite to create its net [36]. So we used the Ca concentration of DKW in $mDKW_2$ to avoid the deficiency symptoms of explants. Morard and Henry [26] also proposed a new mineral composition for *in vitro* culture of *Solanum paludosum* with a larger amount of Ca than that of K.

The explants grown on $mDKW_1$ showed yellow leaves and shoot–tip necrosis. These symptoms are typical for calcium deficiency in tissue cultures [20]. Calcium depletion effects are always visible in young leaves, although photosynthetic efficiency is not affected [4]. It is likely that Ca deficiency also results in decreased growth rate [11]. In a non-circulating nutritive solution, a recombination between $H_2PO_4^-$ and Ca^{2+} is responsible for an insoluble form of calcium. In addition, calcium availability is low in media, because of its low solubility in water [25].

Among the mobile elements, nitrogen generally has the greatest effect upon growth, affecting cell number and cell size; phosphorus has similar but less pronounced effects and potassium has the least effect upon growth, affecting mainly cell size [40]. Considering the macronutrients most changed in mDKW, increased P concentration is a common modification recommended many years ago [27]. Nitrogen is also an important factor in the improvement of the tissue growth of cultures. NH_4^+ could be toxic by a rapid acidification of the medium [19] as we could observe for the multiplication shoot cultures of *S. paludosum* with MS medium.

Phosphate can be a heavily-consumed ion that limits *in vitro* development [25] and supplementation of phosphate can have diverse beneficial effects such as improved regeneration and stabilized growth of cells in continuous culture [7]. However, the high levels of phosphate influence the uptake of other minerals[37]. The strong relationship between medium mineral concentrations and plantlet mineral concentrations *in vitro* may be related with the absence of roots and suggests that diffusion is involved in plantlet mineral uptake *in vitro* [11].

The use of mineral concentration of *in vitro* grown shoots for developing the medium in our study is in agreement with a study on olive [34] where better growth rate was seen in the same approach. Although some researchers [2, 3, 10, 11, 24, 38] used the mineral concentration of plants to promote the healthy plant growth *in vitro*.

According to the results, we can conclude that the growth rate of walnut explants is better in medium adapted to explant mineral concentration because of the better balance of macronutrients. Mineral analysis of healthy plants from the field may be used to influence the initial selection of an *in vitro* medium [11]. Plants cannot produce a high yield without the appropriate nutrition, and healthy plants contain all necessary nutrient elements in near optimal ratios and concentrations [15]. Clearly the optimum balance of minerals in the basal medium is critical in promoting healthy plant growth *in vitro* and in control of growth disorders which are often related to mineral deficiency or toxicity. Tissue analysis was shown to be an essential tool for the diagnosis of mineral imbalances *in vitro* [21]. The lower rooting percentage of plantlets in the $mDKW_2$ than in DKW has also revealed that this medium needs more modifications with subsequent subcultures and changes in the culture medium formulations.

Murphy *et al.* [29] suggested that it might be worthwhile to periodically grow plantlets on different media to neutralize any long-term deterioration induced by continuous growth on a specific medium. However, Ni has been recognized as an essential element for plant growth [1] but is absent from most media formulations. Therefore, the absence of Ni in culture media needs further investigations [11].

Because there were no changes in the plant growth regulators, we could not expect great differences in multiplication, and the improvement found for $mDKW_2$ can be attributed to the greater size of the individual plantlets, so a new balance of growth regulators should be tried in the modified medium. The described method for selection of specific macronutrient composition seems to be a good tool for a better understanding of *in vitro* plant nutrition.

Acknowledgements

The authors thank the University of Tehran and Iran National Science Foundation (INSF) for financial supports and also the analytical chemistry laboratory of College of Sciences, University of Tehran for providing ICP spectroscopy.

References

[1]Brown, P. H., Welch, R. M., Cary, E. E. and Chechai, R. T. 1987. Beneficial effects of nickel on plant growth. J. Plant Nut. **10**:2125-2135.

[2]Birmeta, G. and Welander, M. 2004. Efficient micropropagation of *Ensete ventricosum* applying meristem wounding: A three step protocol. Plant Cell Rep. **23**:277-283.

[3]Bouman, H., Morris, B. and Tiekstra, A. 2001. Development of new tissue culture media, using the relation between mineral composition of plant and medium. Acta Hort. **560**:373-376.

[4]Correia, P. J., Pestana, M. and Martins-Loução, M. A. 2003. Nutrient deficiencies in carob (*Ceratonia siliqua* L.) grown in solution culture. J. Hort. Sci. Biotech. **78**:847-852.

[5]Cornu, D. and Jay-Allemand, C. 1989. Micropropagation of hybrid walnut trees (*Juglans nigra*× *Juglans regia*) through culture and multiplication of embryos. Ann. Sci. For. (Paris) **46**:113-116.

[6]Driver, J. A. and Kuniyuki, A. H. 1984. *In vitro* propagation of paradox walnut rootstock. HortScience. **19**:507-509.

[7]Dunstan, D. I. and Short, K. C. 1977. Improved growth of tissue cultures of the onion, *Allium cepa*. Physiol. Plant. **41**:70-72.

[8]El Badaoui, H., Morard, P. and Henry, M. 1996. Stimulation of the growth and solamargine production by *Solanum paludosum* multiple shoot cultures using a new culture medium. Plant Cell Tiss. Org. Cult. **45**:153-158.

[9]Gamborg, O. L., Miller, R. A. and Ojima, K. 1968. Nutrient requirements of suspension cultures of soybean root cells. Exp. Cell Res. **50**:151-158.

[10]Gonçalves, S., Correia, P. J., Martins-Loução, M. A. and Romano, A. 2005. A new medium formulation for *in vitro* rooting of carob tree based on leaf macronutrients concentrations. Biol. Plant. **49**:277-280.

[11]Gribble, K., Conroy, J. P., Holford, P. and Milhan, P. J. 2002. *In vitro* uptake of minerals by *Gypsophila paniculata* and hybrid eucalypts, and relevance to media mineral formulation. Aust. J. Bot. **50**:713-723.

[12]Grusell, R., Nicasie, C. and Boxus, P. 1995. Regulation of *in vitro* shoot multiplication in Persian walnut by different carbon sources and by ammonium phosphate. Bull. Rech. Agron. Gembloux. **30**:47-53.
[13]Jay-Allemand, C., Capelli, P. and Cornu, D. 1992. Root development of *in vitro* hybrid walnut microcutting in a vermiculite containing gelrite medium. Scientia Hort. **15**:335-342.
[14]Judd, T. S., Attiwill, P. M. and Adams, Ma. 1996. Nutrient concentrations in *Eucalyptus*: A synthesis in relation to differences between taxa, sites and components. In Attiwill, P. M. and Adams, M. K. (eds). Nutrition of Eucalyptus. CSIRO Publishing, Collingwood, pp.123-153.
[15]Karlsen, P. 1986. Practical plant nutrition in glasshouses in Denmark. Acta Hortic. **178**:53-58.
[16]Litvay, J. D., Johnson, M. A., Verma, D., Einspahr, D. and Weyrauch, K. 1981. Conifer suspension culture medium development using analytical data from developing seeds. Institute of Paper Chemistry Technical Paper Series. **115**:1-17.
[17]Llyold, G. and McCown, B. 1980. Commercially-feasible micropropagation of mountain laurel, *Kalmia latifolia*, by use of shoot tip culture. Proc. Int. Plant Prop. Soc. **30**:421-427.
[18]Malavolta, E., Vitti, G. C. and Oliveira, S. A. 1997. Avaliação do estado nutricional das plants: Princĩoios e apliações. 2nd edn. POTAFOS, Piracicaba, 319 p.
[19]Martin, S. M., Rose, D. and Hui, V. 1977. Growth of plant cell (Ipomea) suspension cultures with ammonium as the sole source of nitrogen. Can. J. Bot. **55**:2838-2843.
[20]McCown, B. and Sellmer, J. 1987. General media and vessels suitable for woody plant culture. In Bonga, J. and Durzan, D. (eds). Cell and Tissue Culture in Forestry. Martinus Nijhoff, Dordrecht, pp. 4-16.
[21]McDonald, A. J. S. 1994. Nutrient supply and plant growth. In Lumsden, P. J., Nicholas, J. R. and Davies, W. J. (eds). Physiology, Growth and Development of Plants in Culture. Kluwer Academic Publishers, Dordrecht, The Netherlands, pp. 47-57.
[22]McGranahan, G. H., Driver, J. A. and Tulecke, W. 1987. Tissue culture of *Juglans.* In Bonga, J. M. and Durzan, D. J. (eds). Cell and Tissue Culture in Forestry. Vol. 3. Martinus Nijhoff, The Netherlands, pp. 261-271.
[23]McGranahan, G. H., Leslie, C. and Driver, J. 1988. *In vitro* propagation of mature Persian walnut cultivars. HortScience. **23**:220.
[24]Monteiro, A. C. B., Higashi, E. N., Gonçalves, A. N. and Rodriguez, A. P. M. 2000. A novel approach for the definition of the inorganic medium components for micropropagation of yellow passionfruit (*Passiflora edulis* Sims. *flavicarpa* deg.). *In Vitro* Cell. Dev. Biol. Plant. **36**:527-531.
[25]Morard, P., Fulchery, C. and Henry, M. 1998. Kinetics of mineral nutrient uptake by *Saponaria officinalis* L. suspension cell cultures in different media. Plant Cell Rep. **18**:260–265.
[26]Morard, P. and Henry, M. 1998. Optimization of the mineral composition of *in vitro* culture media. J. Plant Nutr. **21**:1565-1576.
[27]Murashige, T. 1974. Plant propagation through tissue-cultures. Ann. Rev. Plant Physiol. **25**:135-166.
[28]Murashige, T. and Skoog, F. A. 1962. A revised medium for rapid growth and bioassays with tobacco tissue culture. Physiol. Plant. **15**:473-497.
[29]Murphy, K. P., Nicholas, J. R., Leifert, C. and Lumsden, P. J. 1994. Growth and mineral nutrition in micropropagated Delphinium during a subculture period. In Lumsden, P. J., Nicholas, J. R. and Davies, W.J. (eds). Physiology, Growth and Development of Plants in Culture. Kluwer Academic Publishers, Dordrecht, The Netherlands, pp.72-76.
[30]Navatel, J. C. and Bourrain, L. 2001. Plant production of walnut (*Juglans regia* L.) by *in vitro* multiplication. Acta Hort. **544**:465-471.
[31]Nas, M. N. and Read, P. E. 2004. A hypothesis for the development of a defined tissue culture medium of higher plants and micropropagation of hazelnuts. Scientia Hortic. **101**:189-200.
[32]Preece, J. E. 1995. Can nutrient salts partially substitute for plant growth regulators? Plant Cell Tiss. Biotech. **1**:26-37.
[33]Ripetti, V., Kevers, C. L. and Gaspar, T. 1994. Two successive media for the rooting of walnut shoots *in vitro*. Changes in proxidases activity and ethylene production. Adv. Hort. Sci. **8**:29-32.
[34]Rugini, E., 1984. *In vitro* propagation of some olive (*Olea europaea sativa* L.) cultivars with different root-ability, and medium development using analytical data from developing shoots and embryos. Scientia Hortic. **24**:123-124.
[35]Ružić, D., Sarić, M., Cerović, R. and Ćulafić, Lj. 2004. Contents of macronutrients and growth of sweet cherry rootstock *in vitro*. Biol. Plant. **47**:463-465.
[36]Scholten, H. J. and Pierik, R. L. M. 1998. Agar as a gelling agent: Chemical and physical analysis. Plant Cell Rep. **17**:230-235.
[37]Shibli, R. A., Sawwan, J., Swaidat, B. and Tahat, M. 2001. Increased phosphorus mitigates the adverse effects of salinity in tissue culture. Communications in Soil Science and Plant Analysis. **32**:429-440.
[38]Terrer, J. C. and Tomąs, D. F. 2001. Determination of macronutrients to be included in *in vitro* culture media according to leaf concentrations. J. Hortic. Sci. Biotech. **76**:484-488.
[39]Vahdati, K., Leslie, C., Zamani, Z. and McGranahan, G. 2004. Rooting and acclimatization of *in-vitro* grown shoots from three mature Persian walnut cultivars. HortScience. **39**:324-327.
[40]Watson, D. J. 1963. Some features of crop nutrition. In Ivins, J. D. and Milthorpe, F. L. (eds). The Growth of the Potato. Butterworths, London, pp.233-247.
[41]Cheng, T. Y. 1975. Adventitious bud formation in culture of Douglas fir (*Pseudotsuga menziensii* Mirb. Franco). Plant Sci. Lett. **5**:97-102.