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Carbohydrate source and concentration affect *in vitro* growth indices of the selected wild cherry (*Prunus avium* L.) genotype

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Abstract

In vitro shoots of the selected genotype of wild cherry grown in natural forests of Nour (north forests of Iran) were subcultured in Murashige and Skoog medium (1/2 N) containing 1 mgl^{-1} BA and 0.1 mgl^{-1} IBA and three different source of carbohydrates (Sucrose, Glucose and fructose) at three levels (0, 15, 30 and 45 g l^{-1}) for proliferation stage. Mean height of longest shoot, Coefficient of multiplication, as the mean of new shoots formed per micro cutting in each subculture, mean of leaf number, dry weight, minerals percentage (N, P, K and Ca) and sucrose content were measured in shoots after 30 and 45 days. Fructose resulted as the best source of carbohydrate inducing superior rates of growth indices. Higher concentrations, more than 30 g l^{-1}, of each carbohydrate improved the results. The highest sucrose content in shoots was achieved in media supplemented with 45 gl^{-1} sucrose and fructose after 15 and 30 days, respectively. The maximum uptake of N (%) was achieved in shoots treated by 45 g l^{-1} fructose both after 15 and 30 days. It was concluded that type of carbon source affected significantly growth indices, consequently the rate of mineral absorption and levels of carbohydrates of the in vitro shoots at different stages of growth.

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Introduction

Wild cherry trees are deciduous, highly vigorous, 15 to 30 m, thorny shoots with alternate leaves and strong deep root system. This species is commonly diffused in broad leaf forests of northern Iran, the hermaphroditic flowers with five pure white petals borne in corymbs 2 to 6 together are produced in early spring contemporaneously with new leaves. Botanically is known as thermopiles, self unfertile species with low chilling need (Ivanica, 1992). The cortex a gum type exudates', called 'Angom', is highly requested by pharmaceutical industries (Sabeti, 1995). Considering hardy rooting of wild type trees cuttings others proposed propagation by layering (Ivanica, 1992), but seems adaptable to propagate through micropropagation (Ružić *et al.*, 2000; Hammatt and Grant, 1996). *In vitro* shoots need different nutritional sources including minerals and carbohydrates for biological activities and growth (Hajnajari *et al.*, 2009; Miller and Timmer, 1997). *Prunus avium* genotypes, wild types, cultivars and rootstocks, were largely micropropagated, particularly for horticultural proposes (Zilkah *et al.*, 1992; Schmidt and Ketzel, 1992; Troyanos *et al.*, 1997; Grant and Hammat, 1999; Akita *et al.*, 2006; Ďurkovič, 2006). Molecular structure of carbohydrates as the main fount of energy is constituted generally mostly by carbon. A normal metabolism process in living organism during growth is ensured by providing energetic substances. The energy need, specific dose and source, is in function of internal reactions defined by existing meticulous requirements demanded through genetic pattern of each genotype under equal conditions (Emanue and Bloom, 2005). Thus, many studies focused on *in vitro* nutritional needs and the factors affecting absorption and growth to improve micropropagation outcome (Tuija Aronen, 2009). Carbohydrates cause higher osmotic potentials than salts, with direct consequences on water absorption rupture resulting in morphogenesis recess and lowering growth indices of in vitro shoots (Dussert, 1995; Stromberger and Tsai, 1994). Final osmotic potential of the medium is defined by its single constituents like minerals, organics, agar and carbohydrates (Hasanloo *et al.*, 2006). The intent of this experiment was studying the influence of source and dose of fructose, sucrose, glucose on in vitro shoot growth indices forced by further assessment regarding levels of single minerals uptake through in vitro biomass analyzes of the selected wild cherry grown in Iranian broadleaf forests, near Caspian Sea. The excised explants, terminal and lateral buds, were surface sterilized and established in MS medium using 1 mg l^{-1} BA and 0.1 mg l^{-1} IBA (Hajnajari *et al.*, 2009).

Materials and methods

Explant type and medium: The 3 cm long uni-binodal micro cuttings carrying 2-3 primordial leaves were subcultured in modified MS medium, half strength nitrogen compounds (N/2).

Experimental design and treatments: This factorial experiment was conducted within Completely Randomized Design (CRD), with carbohydrate source, fructose- glucose- sucrose, as main factor and concentration, 15- 30- 45 g l^{-1}, as the second factor besides the control carbohydrate free medium.

Data collections and analyses

Data collections were made in bi-weekly intervals, 15 and 30 days after subculturing the samples in the treatments. After 15 days growth indices including shoot height, proliferation coefficient, leaf number, green intensity, and leaf longevity were evaluated and the same characters jointly with fresh and dry weights were measured after 30 days. Levels of three carbohydrate sources and N, P, K and Ca contents were analyzed both in the shoots and remaining part in the medium, in two successive temporal intervals. Colorimetric method, Antron Sulfuric Acid and Flame photometer, were used to measure the carbohydrates, N, P, K and Ca contents using Standard Curve. (Spectrophotometer, Cary 300; Flame photometer, Corning 410 (Brink *et al.*, 1960).

Statistical analysis

The data were given as the mean of at least three replicates. Statistical analysis was performed with SAS software (Version 6.2) using ANOVA method

with Duncan test set at $\dot{\alpha} \leq 0.05$. MSTATC software was used for multivariate analyzes and duncan test.

Results

15 days after subculture, uni-binodal microcuttings *in vitro* shoots of the selected wild cherry, demonstrated no significant difference ($p \leq 0.05$) of mean height in diverse carbohydrate sources at all the three concentration levels, while coefficient pf proliferation (CP), leaf number and leaf longevity showed significant differences ($p \leq 0.05$) (Table 1). Data analyzes of the growth indices after 30 days exhibited ineptitude of treatments for shoot height likewise compared with the precedent temporal measurement, but there were found significant differences in proliferation coefficient caused by unlike carbohydrate source (Table 2). The highest dose of carbohydrates, 45gl^{-1}, increased fresh and dry weights of biomass up to 3.24, 5.35 and 4.63g related to the carbohydrate source (CS) sucrose, glucose and fructose, correspondingly with no influence on CP (Table 2). Statistical analyses showed the higher efficiency of glucose and fructose compared with sucrose and carbohydrate free (CF) control just after 15 days from subculture (Fig. 1). The control treatment (CF), produced the least amount, 0.86 gr., of *In vitro* biomass. It confirmed that soon after sugar depletion, plants begin to decline respiration and proliferation because of growth-associated genes repression (Devaux *et al.*, 2003). The mean comparisons of growth indices of *In vitro* shoots demonstrated that fructose source improved proliferation coefficient (CP) and shoot height (SH) opposing glucose, while the latter source induced advanced level of leaf production and Green intensity, at the end of 15 days. After 30 days, the superiority of fructose and glucose sources was definitively confirmed for all growth indices weighed against sucrose and control. Final comparative observations between fructose and glucose indicated superiority of fructose related to glucose. The fructose source, raised CP and biomass production in terms of fresh and dry matter; thus it could be recommended to be used as the best source of carbohydrate for micropropagation of the selected Iranian wild cherry (Table 1) and probably for other rootstocks and cultivars within the species because of its higher mineral uptake efficiency (Table 3).

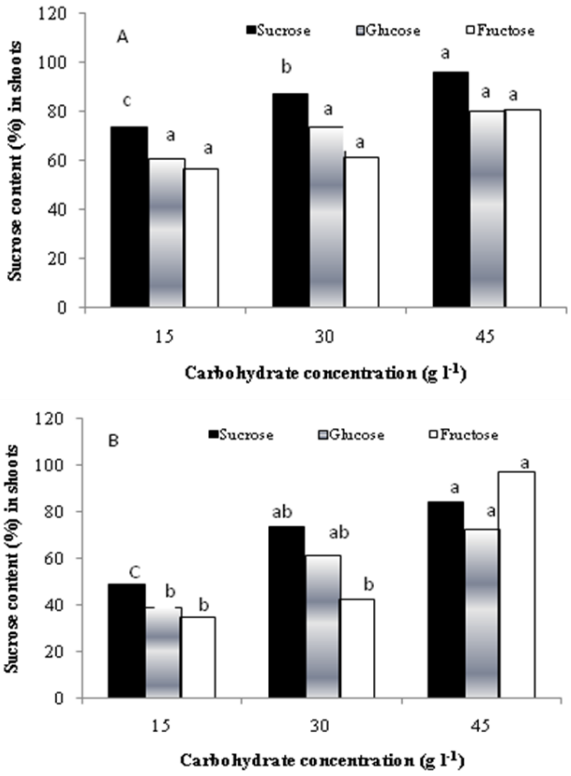

Fig. 1. Sucrose content in *in vitro* shoots of the selected cherry genotype in media supplemented with different carbohydrate source and concentration after 15 (A) and 30 (B) days.

Growth indices comparison made on *in vitro* shoots grown up in different concentrations of sucrose source explained positive enhance in CP and shoot height, but declining effect on Leaf longevity or *in vitro* durability. It was clarified that using higher concentrations of sucrose, 30 and 45gl^{-1}, better growth indices will be attained, at the end of 15 days. In the first 15 days, all under study indices of shoot height, CP and biomass production were promoted in presence of highest fructose dose. Use of fructose and glucose showed better results than fructose, nevertheless no significant difference ($p \leq 0.05$) was found among different concentrations, at the end of 15 days. Considering the target of experiment, by the means of increasing CP and shoot height, becomes evident the priority of the treatments 30 and 45 g l^{-1} of fructose and glucose. In a general view,

concentrations higher than 30 g l^{-1} of all three carbohydrate sources showed better results than lower concentrations. Final mean comparisons regarding dry matters confirmed the existence of direct correlation between increase in carbohydrate concentration and raises in dry matter, except sucrose in which the highest dose, 45gl^{-1}, resulted in metabolic dysfunction decreasing dry matter through an inhibitory roll. Fig.1. Table 1. Table 2. Table 3.

Table 1. The response of *In vitro* shoots of the selected cherry genotype to the carbohydrate sources and concentrations after 15 days.

Carbohydrate source	Carbohydrate concentration (g l^{-1})	Morphological parameters			Dry weight (g)
		Mean height of longest shoot(cm)	Coefficient of multiplication	Mean of leaf number	
Sucrose	15	2.11 cd	1.57 cd	10.19 ab	1.45 d
	30	2.14 bcd	2.00 c	8.31b cd	2.56 c
	45	2.29 abc	2.06 c	7.73 cde	1.95 d
Glucose	15	2.64 a	2.69 b	11.93 a	3.39 abc
	30	2.07 cde	2.97 b	7.69 cde	2.47 c
	45	2.21 abc	2.97 b	6.97 de	3.48 abc
Fructose	15	2.29 abc	2.71 b	9.49 bc	2.30 cd
	30	2.68 a	3.23 b	7.59 cde	3.78 a
	45	2.61 ab	4.03 a	6.82 de	3.58 ab
Carbohydrate Free		1.79 de	1.06 d	5.75 e	0.74 e

Table 2. The response of *In vitro* shoots of the selected cherry genotype to the carbohydrate sources and concentrations after 30 days.

Carbohydrate source	Carbohydrate concentration (g l^{-1})	Morphological parameters			Dry weight (g)
		Mean height of longest shoot (cm)	Coefficient of multiplication	Mean of leaf number	
Sucrose	15	2.58 cd	2.80 ef	9.12 ab	3.27 d
	30	2.83 abc	2.80 ef	8.79 ab	2.81 e
	45	2.67 bc	3.53 de	7.04 ab	3.24 d
Glucose	15	3.00 abc	5.40 bc	11.02 ab	4.82 bc
	30	2.33 cd	6.80 b	11.30 a	5.98 b
	45	3.67 a	5.20 bcd	8.84 ab	5.35 b
Fructose	15	2.33 cd	4.67 cd	9.22 ab	4.79 bc
	30	3.17 abc	9.67 a	8.69 ab	6.75 a
	45	3.50 ab	5.73 bc	7.59 d ab	4.63 bc
Carbohydrate Free		1.50 d	1.13 c	5.82 b	0.86 g

Table 3. N, P, K and Ca percent in *in vitro* shoots of the selected cherry genotype in media supplemented with different carbohydrate sources and concentrations after 15 and 30 days.

Carbohydrate source	Carbohydrate concentration (g l^{-1})	N, P, K and Ca percent after 15 days				N, P, K and Ca percent after 30 days			
		N	P	K	Ca	N	P	K	Ca
Sucrose	15	2.14 cd	0.44 a	3.25 a	1.40 abc	2.04 cd	0.35 a	3.07 a	1.68 ab
	30	2.78 c	0.29 b	2.07 bc	1.72 a	2.31 c	0.35 ab	1.99 c	1.93 a
	45	2.48 c	0.23 bc	2.29 b	1.08 bcd	2.24 c	0.29 a	2.19 abc	1.44 ab
Glucose	15	3.57 ab	0.29 b	3.12 a	1.60 ab	3.15 ab	0.29 a	3.08 a	1.83 a
	30	3.64 ab	0.21 bcd	2.57 b	1.56 ab	3.21 ab	0.23 ab	2.41 ab	1.64 ab
	45	3.91 a	0.18 bcd	2.09 bc	1.16 bc	3.53 a	0.18 b	1.16 cd	1.38 ab
Fructose	15	3.61 ab	0.24 bc	3 ab	1.43 ab	3.27 ab	0.26 ab	2.11 abc	1.54 a
	30	3.67 ab	0.21 b	2.57 b	1.36 bc	3.19 ab	0.18 b	2.98 ab	1.42 ab
	45	3.98 a	0.18 bcd	2.16 d bc	1.04 d cd	3.61 a	0.17 b	1.74 d c	1.28 d ab
Carbohydrate Free		1.50 e	0.33 c	3.13 a	0.44 e	1.28 e	0.27 ab	2.98 ab	0.52 c

Discussion

A factor that must be considered when propagating a plant species *in vitro* is the type of medium to use. The medium is comprised of basal salts and essential nutrients that a plant requires for proper growth and development (Bidarigh and Azarpour, 2011). The carbohydrates are considered as one of the most important constituents in all the substrates (Leifert *et al.*, 1992). Seeing as energy source to grant carbonic structures of living cells for biosynthetic process (Mclachland, 1976). Use of carbohydrates in medium is vital due to the low photon photosynthetic flow in the growth chambers and CO_2 concentration in the vase both may appear as limitant factors of growth to obstacle natural process of photosynthesis in the plant tissues (Leifert *et al.*, 1992; Eapen and Georg, 1990). Normally, sucrose is commonly used for in vitro cultivation, but the concentration and source of carbohydrate may depend on type and age of the growing shoots (Leifert *et al.*, 1992; Eapen and Georg, 1990). The carbohydrates are extracted in various modalities, assays for glucose, fructose and sucrose were achieved (Kunst *et al.*, 1984) and adapted to a micro assay using an MR 5000 reader (Devaux *et al.*, 2003). We analyzed the sucrose both absorbed by the shoots and remaining part in the medium, in two successive intervals. Later, this method was used for micropropagation of *Prunus avium* and coconut (Ružić and Vujović, 2008; Fuentes *et al.*, 2007), so that the carbohydrate level measured in intervals of 0, 20 and 40 days after subculturing, they noted that aside of increased fresh and dry weight in the explants, opposite rhythm occurred in the media. Effects of different carbon sources on shoot proliferation were examined. Glucose provided better shoot proliferation than sucrose, sorbitol and fructose. In the presence of sucrose, leaf chlorosis occurred and shoots gradually declined, similar results were reported in *Prunus mume* (Harada and Murai, 1996). The present research showed higher efficiency of monosaccharide carbohydrates, fructose and glucose than sucrose, just in the first two weeks of establishment. Even though, the growth indices of *in vitro* shoots were markedly improved by the sucrose levels at the end of subculture period, but the other two monosaccharides caused enhanced growth characters. These results coincide with the findings reported for *Solanum eleagnifolium* (Nigra *et al.*, 1990), considering simple molecular structure of fructose and glucose may be absorbed easier than disaccharides. Clearly, total carbohydrate deletion in the control resulted in a poor shoot quality. Though generally the carbohydrates are active in creating osmotic equilibrium in the medium, but exhibited significant differences among different sources of carbohydrates means that disaccharides, perversely to monosaccharides, tend to raise osmotic pressure (Bozena and Szczerba, 1991). It seems that high osmotic pressure exercised by sucrose affects negatively growth indices lowering the mineral uptake and in vitro biomass reduce. It was shown that *In vitro* shoots of wild cherry were adapted well in a lower osmotic pressure, presenting optimum conditions of growth compared with other sources of carbohydrate. Mean comparisons (Table 1 and 2) regarding growth indices demonstrated the prevalence of the medium containing fructose than glucose. This may be attributed both to the easier absorption and metabolic process of Fructose rather than Glucose. After 15 days, the analyses confirmed absorption of substantial amount of carbohydrates sources in all applied doses by the shoots, nevertheless we observed a weak response in growth characteristics and biomass production (Table 1 and 2), it seems that during the initial phase, the microcuttings consume the absorbed carbohydrates for compensation of excised surface, transfer shock, adaptation to new medium conditions and activation of cell differentiation process based on genetic demand and contemporaneously beginning of a low level of photosynthesis, all time and energy consuming. This is considered as preliminary phase for cell division. Furthermore, increase in energetic compounds of the cell sap may play a role in mitotic phase (Dussert, 1995). High respiration rate during the slow phase of growth is also reported in coconut (Dussert, 1995). During the second phase of *in vitro* shoot culture we noted improved growth indices accompanied with higher levels of carbohydrate absorption from the medium (Tables 1 and 2). It can

be related to complete adaptation of in vitro shoots and acceleration of mitotic cell divisions in various tissues and consequently increased respiration level with high requirement to carbohydrate source for biosynthesis of more complex macromolecules. Selective absorption of carbohydrates is regulated genetically. *Prunus cerasus* (Bozena and Szczerba, 1991) resulted in different responses changing carbohydrates source and dose. Even sucrose and glucose favored a similar rate of proliferation, but it was coupled with the highest frequency of long shoots formation. Others reported that among the three carbon sources, sucrose proved to be better for shoot regeneration than fructose or glucose (Baskaran and Jayabalan, 2005; Madhulatha *et al*., 2006). According to the results of the present work on the influence of carbohydrate source on growth indices, also others confirmed the role of these compounds on control of morphogenesis through acting as energy source and by altering the osmotic potential of the medium, which alters cell wall composition, elongation and hardening followed by morphogenesis modification (Pritchard *et al*., 1991).

Conclusions

Increasing carbohydrates concentration up to the highest dose, 45gl^{-1}, fresh and dry weights of biomass were enlarged equal to 3.24, 5.35 and 4.63 g based on the carbohydrate source, sucrose, glucose and fructose, correspondingly with no influence on coefficient of proliferation while the control produced the least amount of biomass. Fructose and glucose presented more enhanced growth indices on *in vitro* shoots of the selected wild cherry genotype compared with sucrose though fructose source improved coefficient of proliferation and shoot height. After 30 days, the superiority of fructose and glucose sources was definitively confirmed for all growth indices weighed against sucrose and control. Generally, concentrations higher than 30gl^{-1} for all sources of carbohydrate showed better results than lower concentrations. Concluding comparisons between fructose and glucose indicated superiority of fructose. It could be proposed either for micropropagation of other rootstocks and cultivars within the species as the best source of carbohydrate because of its higher mineral uptake efficiency.

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