DOI: 10.17707/AgricultForest.62.3.01

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MINERAL STRENGTH, SUCROSE LEVEL AND MANNITOL CONCENTRATION EFFECTS ON CHERRY ROOTSTOCKS MICROPROPAGATION

SUMMARY

The aim of the present study was to investigate the effects of two strength media in inorganics (full and half), two sucrose levels (15 and 30 g/l) and four mannitol concentrations (0, 5, 10, 20 g/l) in combination with 1 mg/l indole-3-butyric acid (IBA) on the morphogenic and biochemical responses in CAB-6P and Gisela 6 cherry rootstocks. In CAB-6P, root number (7.4) and rooting percentage (83.33%) were maximum in half MS medium supplemented with 5 g/l mannitol and 15 g/l sucrose. Root length was greatest (40.6 mm) with 10 g/l mannitol and 30 g/l sucrose in full MS medium. In Gisela 6, in full MS medium, 20 g/l mannitol + 15 g/l sucrose exhibited the maximum root number (6.88), while 10 g/l mannitol + 30 g/l sucrose gave the greatest root length (50.3 mm). Rooting percentage was highest (92.31%) in half MS + 15 g/l sucrose (mannitol-free) and in 10 g/l mannitol + 15 g/l sucrose (full MS) combination treatments. In CAB-6P, mannitol led to depleted chlorophyll, carotenoid and porphyrin levels in half MS medium for both sucrose levels. Mannitol resulted in elevated leaf and root carbohydrate as well as proline levels irrespective sucrose level and medium strength. In Gisela 6, mannitol + 15 g/l sucrose decreased carotenoid content (full MS) and increased leaf proline content (half MS). In roots, 10 g/l mannitol raised proline (full MS) and carbohydrate content (half MS) in both sucrose levels. Leaf carbohydrate content was higher in half MS medium supplemented with 30 g/l sucrose. In both rootstocks, higher chlorophyll levels were recorded in half MS medium supplemented with 15 g/l sucrose compared to the full MS one or with 30 g/l sucrose. In full MS medium, increase of sucrose concentration led to depleted proline levels in Gisela 6 leaves and CAB-6P roots indicating activation of osmoregulation and osmotic adjustment mechanisms located in leaves for Gisela 6 and in roots for CAB-6P. An efficient root regeneration protocol and biochemical status evaluation of micropropagated cherry rootstocks shoot tips under the combined influence of different strength media, sucrose and mannitol concentrations was established.

Keywords: carbohydrates, *in vitro* rooting, mannitol, mineral salt composition, photosynthetic pigments, proline.

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Notes: The authors declare that they have no conflicts of interest. Authorship Form signed online.

INTRODUCTION

Growth and root initiation are high energy requiring processes that can only occur at the expense of available metabolic substrates, which are mainly carbohydrates (Calamar and De Klerk 2002). The establishment of effective shoot proliferation and root development in vitro is essential for subsequent success during acclimatization to autotrophic conditions (Premkumar et al. 2003). The addition of a carbon source in any nutrient medium is essential for in vitro growth and development of many species, because photosynthesis is insufficient, due to the growth taking place in conditions unsuitable for photosynthesis or without photosynthesis (in darkness) (Pierik, 1997).

In general, sucrose is the carbohydrate of choice as carbon source for in vitro plant culture, probably because it is the most common carbohydrate in the phloem sap of many plants (Fuentes et al., 2000) and because is cheap, readily available, relatively stable to autoclaving, and readily assimilated by plants (Fowler, 2000). It has also been well documented that certain plant tissues may contain and/or utilize different carbohydrates at the same time. It is then not surprising that carbon sources other than sucrose might be effective in promoting in vitro tissue specific growth responses in a given species (Swedlund and Locy 1993).

There are a number of species however that can grow on carbohydrates different than sucrose such as sorbitol, glycerol or mannitol (Vu et al. 1993).

In olive, mannitol is a major product of photosynthesis that reaches high concentrations in leaves that are second only to those of glucose and is translocated in the phloem (Flora and Madore 1993). Leva et al. (1995) have reported that mannitol improved the in vitro propagation of agamic olive explants, collected from mature trees growing in the field.

According to Ibrahim (1999), the concentration of inorganic salts plays an important role in root induction as it was shown that the reduction of MS salts strength to ¾ of the original concentration stimulated root formation in date palm tissue culture. The mineral concentration of the culture medium affects rooting characteristics, and some researchers have proposed its reduction to half normal strength for rooting improvement (Dimassi-Theriou and Economou 1993).

Therefore, the objectives of this study was to evaluate the effects of mannitol concentration, medium composition in minerals and sucrose level under in vitro conditions on the rooting process, vegetative growth, callusing traits as well as on biochemical parameters such as photosynthetic pigments, carbohydrate metabolism and endogenous free proline accumulation in two cherry rootstocks, namely CAB-6P and Gisela 6.

MATERIAL AND METHODS

Plant material and culture conditions

In this experiment, the effects of two different strength media in inorganics; full and half, two different sucrose levels; 15 and 30 g/l and four

different mannitol concentrations; 0, 5, 10 and 20 g/l in combination with 1 mg/l IBA on vegetative growth, rooting and callusing of CAB-6P (Prunus cerasus) and Gisela 6 (Prunus canescens x Prunus cerasus) cherry rootstocks were studied. Shoot tips, 1.5 to 2.5 cm long were obtained from previous in vitro subcultures and placed onto a MS (Murashige and Skoog 1962) culture medium. The pH value of the culture medium was adjusted to 5.8 and 6 g/l agar was added prior to autoclaving at 121 oC for 20 min. The experiment was comprised of 16 treatments and each treatment included 13 replications for CAB-6P and 22 replications for Gisela 6 with one microcutting placed in each 25x100 mm glass test tube with flat base that contained 10 ml of the nutrient culture medium. Afterwards, the cultures were maintained at 21-23 oC under cool white fluorescent light (Phillips, 90 μmol/m2/s) with a 16-hour photoperiod. After six weeks of culture, records and measurements were taken for root number per rooted explant, root length (mm), root fresh weight (g), rooting percentage (%), shoot length and fresh weight of the initial explant (shoot tip without roots), callus fresh weight, callus induction frequency (%), total leaf chlorophyll (a+b), carotenoid and porphyrin content, total carbohydrate and endogenous proline content in both leaves and roots.

Chlorophyll determination

For chlorophyll measurement, 0.1 g of frozen leaf material was taken and placed in glass test tubes of 25 ml volume. Fifteen ml of 96% ethanol was added in each tube, which was covered with aluminum foil to reduce ethanol evaporation. The tubes were incubated in a water bath of 79.8 oC, until complete sample discoloration and chlorophyll extraction. After chlorophyll extraction, the samples (tubes) were allowed to cool at room temperature and the level of 96% ethanol was completed to be 15 ml volume. The absorbance of chlorophylls a and b was measured at 665 and 649 nm, respectively, in a visible spectrophotometer. Chlorophyll concentration was determined according to Wintermans and De Mots (1965) from the following equations:

$$\text{Chl (a + b)} = (6.10 \times A665 + 20.04 \times A649) \times 15/1000/FW \text{ (mg/g FW)}$$

$$\text{Chl (a + b)} = (6.10 \times A665 + 20.04 \times A649) \times 15/1000/DW \text{ (mg/g DW)}$$

Proline determination

Leaf or root frozen tissue (0.1g), was chopped into small pieces and placed in glass test tubes of 25 ml. In each tube, 10 ml of 80% (v/v) ethanol was added and placed in a water bath of 60 oC for 30 min (Khan et al. 2000). The tubes were covered with aluminum foil to reduce evaporation. The extracts were filtered and 80% (v/v) ethanol was added until the total volume (ethanol extract) to be 15 ml. After extraction, the aluminum foil was removed and the tubes were allowed to cool at room temperature. In each tube, 4 ml of toluene was added and mixed well with a vortex. Two layers were visible in each tube. The supernatant (toluene layer) was removed with a pasteur pipette and was placed in a glass

cuvette. The optical density of the extract was measured at 518nm. The extract was filtered with Whatman No. 1 filter paper and free proline was measured (Troll and Lindsley 1955) with acid ninhydrin solution. Proline concentrations were calculated from a standard curve by using L-proline (Sigma Chemical Company) at 0-0.2 mM concentrations.

Carbohydrate determination

Carbohydrate determination of plant tissue was conducted by using the anthrone method (Plummer, 1987). For reagent preparation, 1g of anthrone was diluted to 500 ml concentrated sulfuric acid (96%). The extract (plant ethanolic extract) for carbohydrate determination was the same as that used for proline, with the only difference that it was diluted 10 times with 80% (v/v) of ethanol. In each test tube, 2 ml of anthrone reagent were placed and maintained in an ice bath. Subsequently, the diluted extract (10% of the initial) was added dropwise in contact with the test tube walls in order to avoid blackening of the samples. After shaking the tubes with a vortex, the samples were incubated in a water bath of 95 oC for 15 min. Afterwards, the tubes were placed in a cold water bath for cooling and optical density was measured at 625nm. Carbohydrate concentrations were calculated from a standard curve by using 0-0.2 mM sucrose concentrations.

Carotenoid and porphyrin content measurement

Carotenoid and porphyrin concentrations were determined as decribed Lichenthaler (1987) and Porra et al. (1989) and modified by Yang et al. (1998). Five milligrams of samples were homogenized with 5 ml of 80% acetone in a cooled mortar. Extract was centrifuged for 5 min at 1,500 g, and the supernatant was stored. The pellet was re-extracted with acetone and centrifuged again. This process was continued until the supernatant was colourless, and then the supernatant was pooled.

1. Absorbance was measured at 663.6, 646.6 and 440.5 nm, the major absorption peaks of chlorophyll a and b and carotenoids, respectively. Carotenoids were calculated using the following equation: (4.69 × A440.5 - 1.96 × A663.6 - 4.74 × A646.6) x volume of supernatant (ml) × dilution factor / sample weight (g).

2. Absorbance was measured at 663.6, 646.6, 440.5, 575, 590 and 628 nm, the absorption peaks of chlorophyll a, chlorophyll b, carotenoids, protoporphyrin, magnesium-protoporphyrin and protochlorophyllide, respectively. Porphyrin content was summed (A+B+C) by the following three equations:

A = [(12.25 × A663.6 - 2.55 × A646.6) × volume of supernatant (ml) × diluted factor / sample weight (g)] / 892 × 1000

B = [(20.31 × A646.6 - 4.91 × A663.6) × volume of supernatant (ml) × diluted factor / sample weight (g)] / 906 × 1000

C = [(196.25 × A575 - 46.6 × A590 - 58.68 × A628) + (61.81 × A590 - 23.77 × A575 - 3.55 × A628) + (42.59 × A628 - 34.32 × A575 - 7.25 × A590)] × volume of supernatant (ml) × dilution factor / sample weight (g).

Statistical analysis

The experimental layout was completely randomized and the experiment was repeated twice. Thus, the reported data are the means of the two experiments. The means were subjected to analysis of variance (ANOVA) and compared using the Duncan multiple-range test ($P<0.05$).

The experiment was a 2x2x4 factorial with two different strength media in inorganics (full and half), two different sucrose levels (15 and 30 g/l) and four mannitol concentrations (0, 5, 10, 20 g/l) for each of the two studied cherry rootstocks (CAB-6P and Gisela 6). The main effect of factors (medium strength in inorganics, sucrose level and mannitol concentration) and their interactions for each cherry rootstock were determined by the General Linear Model (3-way ANOVA).

RESULTS AND DISCUSSION

Effect of medium strength in inorganics, sucrose level and mannitol concentration on rooting, vegetative growth and callus induction characteristics.

In CAB-6P, best rooting results in terms of root number per rooted explant (7.4), root fresh weight (0.073 g) and rooting percentage (83.33%) were obtained with 5 g/l mannitol and 15 g/l sucrose in half strength medium (Figure 1n). On the other hand, root length reached its maximum value (40.6 mm) with 10 g/l mannitol and 30 g/l sucrose in full strength medium (Table 1, Figure 1c). Regarding the vegetative growth of the explants, 5 g/l mannitol and 30 g/l sucrose in full strength medium gave the maximum shoot length (22.31 mm) (Figure 1b) whereas better results for shoot fresh weight were recorded in the absence of mannitol (Figure 1a). Callus fresh weight was maximum (0.170 g) when 5 g/l mannitol was incorporated along with 30 g/l sucrose into the full strength medium in inorganics. Callus induction frequency reached its maximum value (100%) by adding 10 g/l mannitol to the medium containing 30 g/l sucrose irrespective of media composition in mineral salts (Figures 1c, 1k). The same maximum callus induction frequency (100%) was achieved in half MS medium with 15 g/l sucrose in the absence of mannitol (Table 2, Figure 1m).

In Gisela 6, 20 g/l mannitol + 15 g/l sucrose in full MS medium exhibited the greatest root number per rooted explant (6.88) (Figure 2h). Root length and root fresh weight were maximum (50.3 mm and 0.134 g) with 10 or 20 g/l mannitol + 30 g/l sucrose in full MS medium, respectively (Figures 2c, 2d). Rooting percentage was highest (92.31%) with 10 g/l mannitol + 15 g/l sucrose in full MS medium (Figure 2g) as well as in the absence of mannitol with 15 g/l sucrose in half MS medium (Table 1, Figure 2m).

Figure 1 Effect of MS medium strength in inorganics, sucrose level and mannitol concentration combined with 1 mg/l IBA on in vitro rooting of CAB-6P explants: (a) Full MS + 30 g/l sucrose, (b) Full MS + 30 g/l sucrose + 5 g/l mannitol, (c) Full MS + 30 g/l sucrose + 10 g/l mannitol, (d) Full MS + 30 g/l sucrose + 20 g/l mannitol, (e) Full MS + 15 g/l sucrose, (f) Full MS + 15 g/l sucrose + 5 g/l mannitol, (g) Full MS + 15 g/l sucrose + 10 g/l mannitol, (h) Full MS + 15 g/l sucrose + 20 g/l mannitol, (i) half MS + 30 g/l sucrose (j) half MS + 30 g/l sucrose + 5 g/l mannitol, (k) half MS + 30 g/l sucrose + 10 g/l mannitol, (l) half MS + 30 g/l sucrose + 20 g/l mannitol, (m) half MS + 15 g/l sucrose, (n) half MS + 15 g/l sucrose + 5 g/l mannitol, (o) half MS + 15 g/l sucrose + 10 g/L mannitol, (p) half MS + 15 g/l sucrose + 20 g/l mannitol.

Table 1. Effect of medium strength in inorganics, sucrose level, mannitol concentration and their interactions in a medium containing 1 mg/l IBA on CAB-6P and Gisela 6 cherry rootstocks rooting characteristics.

Strength MS In inorganics	Sucrose (g/l)	Mannitol (g/l)	Root number /rooted explant	Root length (mm)	Root fresh weight (g)	Rooting percentage (%)
Rootstock: CAB-6P						
full	30	0	2.79 abc	29.93 cd	0.050 abcd	70.00 h
	30	5	2.43 ab	31.63 d	0.038 ab	53.85 c
	30	10	1.71 a	40.60 e	0.032 a	38.46 a
	30	20	3.17 abc	26.46 bcd	0.037 ab	53.85 c
	15	0	4.13 bcd	31.67 d	0.068 de	80.00 j
	15	5	4.43 cd	22.00 bc	0.042 abc	53.85 c
	15	10	5.13 de	25.34 bcd	0.055 bcde	66.67 f
	15	20	2.71 abc	32.61 d	0.061cde	53.85 c
½	30	0	3.30 abc	19.35 ab	0.036 ab	76.92 i
	30	5	3.17 abc	22.08 bc	0.038 ab	46.15 b
	30	10	2.50 ab	11.33 a	0.030 a	61.54 e
	30	20	3.33 abc	18.58 ab	0.037 ab	46.15 b
	15	0	6.44 ef	19.38 ab	0.052abcde	69.23 g
	15	5	7.40 f	20.86 b	0.073 e	83.33 k
	15	10	4.40 cd	18.35 ab	0.051 abcd	55.56 d
	15	20	3.00 abc	20.84 b	0.035 ab	38.46 a
3-wayANOVA						
Strength MS (A)			0.000*	0.363 ns	0.000***	0.000***
Sucrose level (B)			0.018*	0.000***	0.089 ns	0.000***
Mannitol concentration (C)			0.001**	0.147 ns	0.003**	0.000***
(A)*(B)			0.833 ns	0.044*	0.535 ns	0.000***
(A)*(C)			0.041*	0.158 ns	0.421 ns	0.000***
(B)*(C)			0.044*	0.295 ns	0.371 ns	0.000***
(A)*(B)*(C)			0.014*	0.003**	0.139 ns	0.000***
Rootstock: Gisela 6						
full	30	0	3.87 b	36.29 e	0.052 ab	68.18 g
	30	5	3.75 ab	36.81 e	0.066 abc	30.77 a
	30	10	4.33 bc	50.30 f	0.088 cd	42.86 d
	30	20	5.43 cd	34.13 de	0.134 f	58.33 f
	15	0	5.29 cd	28.27 bcd	0.045 a	77.27 h
	15	5	5.80 de	26.10 abc	0.119 ef	76.92 h
	15	10	3.75 ab	29.63 cd	0.081 bcd	92.31 i
	15	20	6.88 e	23.70 abc	0.100 de	66.67 g
½	30	0	3.40 ab	21.71 a	0.049 a	38.46 bc
	30	5	3.11 ab	23.24 ab	0.049 a	69.23 g
	30	10	2.40 a	23.40 abc	0.041 a	41.67 cd
	30	20	3.00 ab	32.83 de	0.083 cd	53.85 e
	15	0	5.42 cd	24.01 abc	0.096 cde	92.31 i
	15	5	5.80 de	22.60 ab	0.100 de	35.71 b
	15	10	3.64 ab	26.44 abc	0.067 abc	64.71 g
	15	20	5.33 cd	20.51 a	0.087 cd	69.23 g
3-wayANOVA						
Strength MS (A)			0.000***	0.000***	0.001**	0.001**
Sucrose level (B)			0.000***	0.000***	0.004**	0.001**
Mannitol concentration (C)			0.000***	0.000***	0.000***	0.001**
(A)*(B)			0.028*	0.000***	0.002**	0.001**
(A)*(C)			0.009**	0.014*	0.000***	0.001**
(B)*(C)			0.016*	0.000***	0.000***	0.001**
(A)*(B)*(C)			0.744 ns	0.000***	0.215 ns	0.001**

Means denoted by the same letter in each column and for each rootstock separately are not significantly different according to Duncan's multiple range test at $P \leq 0.05$. ns – non significant effect ($P \geq 0.05$). Significant effects at $P \leq 0.01$ (**) or 0.001 (***) according to 3-way ANOVA (n = 13 for CAB-6P and n = 22 for Gisela 6).

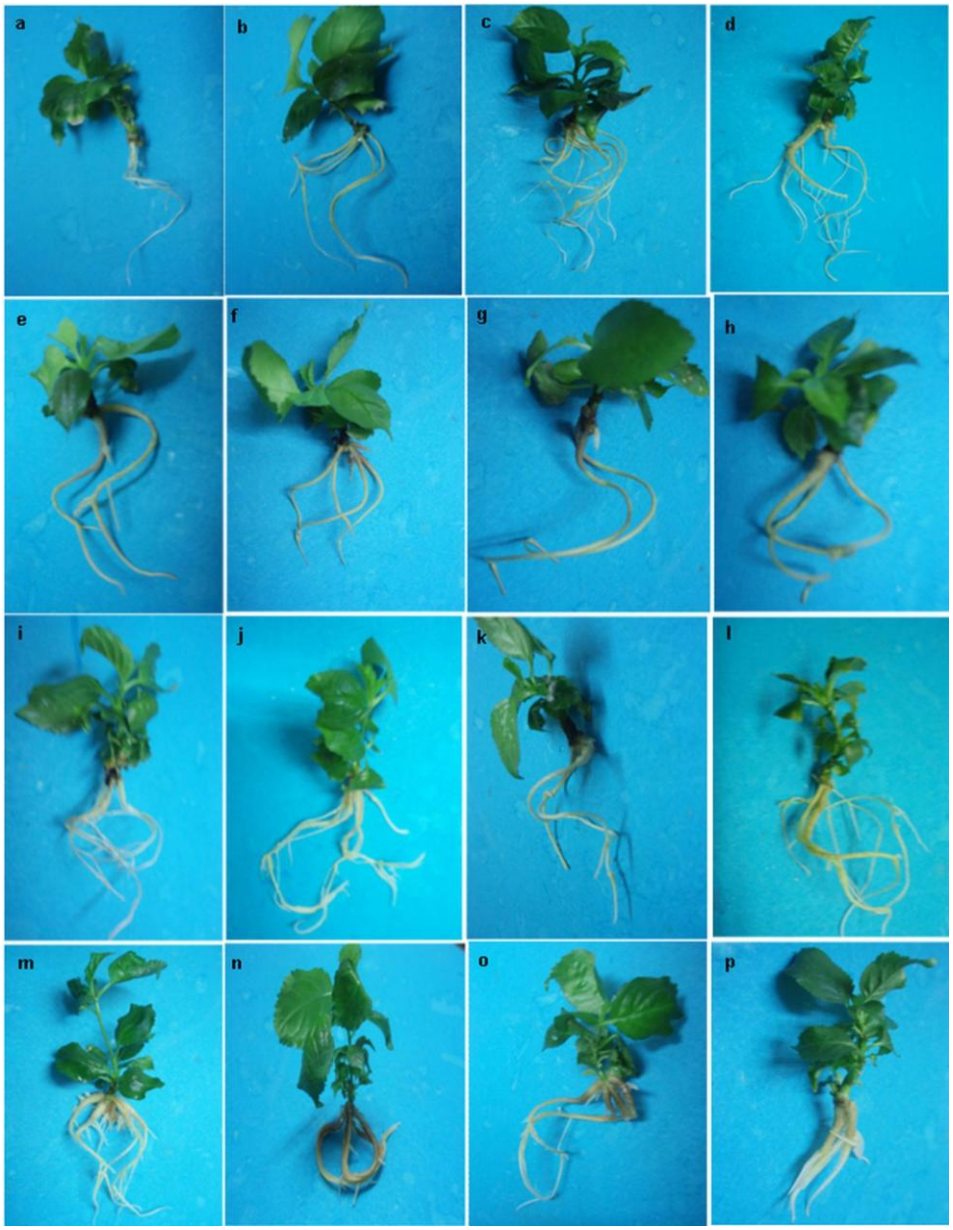

Figure 2 Effect of MS medium strength in inorganics, sucrose level and mannitol concentration combined with 1 mg/l IBA on in vitro rooting of Gisela 6 explants: : (a) Full MS + 30 g/l sucrose, (b) Full MS + 30 g/l sucrose + 5 g/l mannitol, (c) Full MS + 30 g/l sucrose + 10 g/l mannitol, (d) Full MS + 30 g/l sucrose + 20 g/l mannitol, (e) Full MS + 15 g/l sucrose, (f) Full MS + 15 g/l sucrose + 5 g/l mannitol, (g) Full MS + 15 g/l sucrose + 10 g/l mannitol, (h) Full MS + 15 g/l sucrose + 20 g/l mannitol, (i) half MS + 30 g/l sucrose (j) half MS + 30 g/l sucrose + 5 g/l mannitol, (k) half MS + 30 g/l sucrose + 10 g/l mannitol, (l) half MS + 30 g/l sucrose + 20 g/l mannitol, (m) half MS + 15 g/l sucrose, (n) half MS + 15 g/l sucrose + 5 g/l mannitol, (o) half MS + 15 g/l sucrose + 10 g/L mannitol, (p) half MS + 15 g/l sucrose + 20 g/l mannitol.

Full strength medium in inorganics with 30 g/l sucrose in the absence of mannitol (24.09 mm) (Figure 2a), full strength medium with 15 g/l sucrose + 20 g/l mannitol (23.33 mm) (Figure 2h) and half strength medium with 15 g/l sucrose + 5 g/l mannitol (23.21 mm) (Figure 2p) gave better results concerning shoot length.

In the absence of mannitol, half MS medium with 15 g/l sucrose resulted in the maximum shoot (0.190 g) and callus fresh weight (0.120 g). Callus induction frequency was maximum (59.09%) in full MS medium supplemented with 30 g/l sucrose without mannitol (Table 2).

Effect of medium strength in inorganics, sucrose level and mannitol concentration on biochemical parameters

In CAB-6P, half MS medium + 15 g/l sucrose (mannitol-free) and half MS medium + 30 g/l sucrose + 5 g/l mannitol exhibited the highest chlorophyll content.

Carotenoid and porphyrin content were greatest with 30 g/l sucrose in half MS medium in devoid of mannitol (Table 3).

Adding 10 g/l mannitol to the half MS medium with 30 g/l sucrose led to elevated leaf carbohydrate levels. Proline content in leaves was maximum by applying 10 g/l mannitol to full MS medium, irrespective sucrose level. In roots, 5 or 10 g/l mannitol combined with 15 g/l sucrose in full MS medium gave higher carbohydrate and proline content, respectively (Table 4).

In Gisela 6, chlorophyll content was higher by incorporating 10 g/l mannitol + 30 g/l sucrose or 15 g/l sucrose without mannitol into the half MS medium. Treatment of explants in full MS medium supplemented with 15 g/l sucrose resulted in the highest carotenoid content. On the other hand, medium strength in inorganics, sucrose level and mannitol concentration did not influence porphyrin content significantly (Table 3). Mannitol applied at 10 g/l in half MS medium containing 30 g/l sucrose led to elevated levels of carbohydrates in both leaves and roots. Leaf proline levels were higher by incorporating 5 or 10 g/l mannitol into the 15 g/l sucrose containing full MS medium. In the 10 g/l mannitol+15 g/l sucrose+ full MS medium treatment, free root proline was remarkably increased (Table 4).

In both rootstocks, the 2-fold increase in sucrose level from 15 to 30 g/l in both full and half MS media and in all mannitol concentrations resulted in higher root number. Similar results were obtained by Schneider (2005). In specific, half MS + 15 g/l sucrose + 5 g/l mannitol was the optimum treatment for root number and rooting percentage of CAB-6P microcuttings whereas the full MS + 15 g/l sucrose + 20 g/l mannitol treatment gave the highest root number in Gisela 6 explants. In the peach rootstock GF 677, 30 g/l sucrose gave better results regarding rooting percentage and root number, while lower (15 g/l) and higher (45 and 60 g/l) concentrations had an inhibitory effect (Ahmad *et al.* 2007). Manzanera and Pardos (1990) found that rooting percentage and root number were enhanced with increasing sugar concentration. Therefore, the tendency to form roots under the highest sugar concentration may indicate an increase in water demands of these tissues.

Table 2. Effect of medium strength in inorganics, sucrose level, mannitol concentration and their interactions in a medium containing 1 mg/l IBA on CAB-6P and Gisela 6 cherry rootstocks on vegetative growth and callus induction characteristics.

Strength MS in inorganics	Sucrose (g/l)	Mannitol (g/l)	Shoot length (mm)	Shoot fresh weight (g)	Callus fresh weight (g)	Callus induction frequency (%)
Rootstock: CAB-6P						
full	30	0	16.50 abc	0.124 f	0.070 abc	90.00 h
	30	5	22.31 d	0.060 ab	0.085 bc	84.62 g
	30	10	18.85 cd	0.056 a	0.089 bc	100 j
	30	20	15.38 abc	0.071 ab	0.070 abc	61.54 d
	15	0	15.50 abc	0.111 def	0.048 a	45.00 b
	15	5	18.46 bcd	0.090 bcde	0.106 cd	84.62 g
	15	10	17.50 abc	0.070 ab	0.073 abc	83.33 f
	15	20	15.00 abc	0.084 abcd	0.068 ab	23.08 a
½	30	0	17.31 abc	0.079 abc	0.125 de	92.31 i
	30	5	18.08 bc	0.071 ab	0.170 f	92.31 i
	30	10	17.69 abc	0.066 ab	0.142 ef	100 j
	30	20	15.38 abc	0.070 ab	0.087 bc	83.33 f
	15	0	15.77 abc	0.081 abc	0.079 abc	100 j
	15	5	17.08 abc	0.116 ef	0.091 bc	58.33 c
	15	10	13.33 a	0.066 ab	0.090 bc	44.44 b
	15	20	14.23 ab	0.070 ab	0.084 bc	76.92 e
3-wayANOVA						
Strength MS (A)			0.017*	0.001**	0.000***	0.000***
Sucrose level (B)			0.030*	0.635 ns	0.000***	0.000***
Mannitol concentration (C)			0.031*	0.000***	0.029*	0.000***
(A)*(B)			0.932 ns	0.273 ns	0.003**	0.000***
(A)*(C)			0.286 ns	0.073 ns	0.334 ns	0.000***
(B)*(C)			0.012*	0.001**	0.071 ns	0.000***
(A)*(B)*(C)			0.848 ns	0.644 ns	0.046*	0.000***
Rootstock: Gisela 6						
full	30	0	24.09 f	0.181 cd	0.046 cd	59.09 h
	30	5	19.62 bcde	0.171 bcd	0.093 j	7.69 b
	30	10	19.64 bcde	0.181 cd	0.077 h	7.14 b
	30	20	20.00 cde	0.169 bcd	0.080 h	8.33 b
	15	0	22.27 ef	0.149 abc	0.039 b	36.36 e
	15	5	23.08 f	0.181 cd	0.048 d	7.69 b
	15	10	16.54 ab	0.140 ab	0.055 e	7.69 b
	15	20	23.33 f	0.147 abc	0.040 bc	16.67 c
½	30	0	18.08 abc	0.179 cd	0.044 bcd	23.08 d
	30	5	18.46 abcd	0.173 bcd	0.086 i	46.15 g
	30	10	20.00 cde	0.171 bcd	0.070 g	25.00 d
	30	20	15.77 a	0.148 abc	0.061 f	15.38 c
	15	0	21.54 def	0.190 d	0.120 k	7.69 b
	15	5	23.21 f	0.152 abc	0.067 g	35.71 e
	15	10	19.12 bcd	0.129 a	0.041 bc	41.18 f
	15	20	18.85 bcd	0.119 a	0.000 a	0 a
3-wayANOVA						
Strength MS (A)			0.002**	0.000***	0.000***	0.000***
Sucrose level (B)			0.001**	0.189 ns	0.178 ns	0.000***
Mannitol concentration (C)			0.000***	0.001**	0.000***	0.000***
(A)*(B)			0.028*	0.934 ns	0.000***	0.063 ns
(A)*(C)			0.000***	0.099 ns	0.000***	0.000***
(B)*(C)			0.000***	0.036*	0.000***	0.000***
(A)*(B)*(C)			0.229 ns	0.117 ns	0.000***	0.000***

Means denoted by the same letter in each column and for each rootstock separately are not significantly different according to Duncan's multiple range test at $P \leq 0.05$. ns – non significant effect ($P \geq 0.05$). Significant effects at $P \leq 0.001$ (***) according to 3-way ANOVA (n = 13 for CAB-6P and n = 22 for Gisela 6).

Table 3 Effect of medium strength in inorganics, sucrose level, mannitol concentration and their interactions in a medium containing 1 mg/1 IBA on CAB-6P and Gisela 6 cherry rootstocks total leaf chlorophyll, carotenoid and porphyrin content.

Strength MS in inorganics	Sucrose (g/l)	Mannitol (g/l)	Chl(a+b) (mg/g F.W.)	Carotenoids (mg/g F.W.)	Porphyrins (mg/g F.W.)
Rootstock: CAB-6P					
full	30	0	18.969 defg	0.358 abcd	5.990 cdef
	30	5	14.739 abcde	0.460 de	5.794 bcde
	30	10	11.934 abcd	0.339 abc	5.242 abc
	30	20	19.294 defg	0.389 abcd	6.726 fg
	15	0	15.068 bcde	0.411 bcde	5.451 abc
	15	5	19.563 defg	0.285 a	6.402 def
	15	10	9.716 ab	0.336 abc	5.004 a
	15	20	21.550 efg	0.386 abcd	6.893 g
½	30	0	17.755 cde	0.640 f	8.410 h
	30	5	22.879 fg	0.323 ab	6.217 defg
	30	10	15.177 bcde	0.517 e	6.753 g
	30	20	7.267 a	0.377 abcd	5.832 bcde
	15	0	25.725 g	0.451 cde	6.813 g
	15	5	13.733 abcd	0.364 abcd	5.662 abcd
	15	10	17.627 cdef	0.413 bcde	6.481 efg
	15	20	10.918 abc	0.343 abc	5.178 ab
3-wayANOVA					
Strength MS (A)			0.599 ns	0.000***	0.003**
Sucrose level (B)			0.000***	0.074 ns	0.000***
Mannitol concentration (C)			0.000***	0.000***	0.000***
(A)*(B)			0.590 ns	0.223 ns	0.505 ns
(A)*(C)			0.306 ns	0.045*	0.001**
(B)*(C)			0.083 ns	0.327 ns	0.042*
(A)*(B)*(C)			0.648 ns	0.069 ns	0.147 ns
Rootstock: Gisela 6					
full	30	0	18.245 bc	0.355 abc	7.057 a
	30	5	17.053 abc	0.374 abc	6.756 a
	30	10	12.301 a	0.298 abc	5.270 a
	30	20	13.941 ab	0.316 abc	5.897 a
	15	0	16.844 abc	0.451 c	7.159 a
	15	5	11.391 a	0.418 bc	6.937 a
	15	10	14.863 abc	0.277 ab	5.356 a
	15	20	12.830 ab	0.266 ab	5.316 a
½	30	0	17.202 abc	0.370 abc	6.917 a
	30	5	12.352 a	0.234 a	4.839 a
	30	10	20.169 c	0.346 abc	6.861 a
	30	20	16.706 abc	0.419 bc	6.558 a
	15	0	20.464 c	0.413 bc	7.231 a
	15	5	13.182 ab	0.333 abc	5.622 a
	15	10	14.849 abc	0.423 bc	6.800 a
	15	20	18.618 bc	0.356 abc	6.458 a
3-wayANOVA					
Strength MS (A)			0.000***	0.003**	0.004**
Sucrose level (B)			0.897 ns	0.379 ns	0.743 ns
Mannitol concentration (C)			0.236 ns	0.495 ns	0.490 ns
(A)*(B)			0.410 ns	0.184 ns	0.146 ns
(A)*(C)			0.730 ns	0.439 ns	0.679 ns
(B)*(C)			0.052 ns	0.120 ns	0.727 ns
(A)*(B)*(C)			0.805 ns	0.758 ns	0.880 ns

Means denoted by the same letter in each column and for each rootstock separately are not significantly different according to Duncan's multiple range test at $P \leq 0.05$. ns – non significant effect ($P \geq 0.05$) according to 3-way ANOVA (n = 13 for CAB-6P and n = 22 for Gisela 6).

Table 4 Effect of medium strength in inorganics, sucrose level, mannitol concentration and their interactions in a medium containing 1 mg/l IBA on CAB-6P and Gisela 6 cherry rootstocks total carbohydrate and proline content in leaves and roots.

Strength MS in inorganics	Sucrose (g/l)	Mannitol (g/l)	Leaf carbohydrates (μmol/g F.W.)	Leaf proline (μmol/g F.W.)	Root carbohydrates (μmol/g F.W.)	Root proline (μmol/g F.W.)
Rootstock: CAB-6P						
full	30	0	51.207 a	2.850 a	42.030 a	0.936 a
	30	5	76.033 cd	3.016 a	83.304 d	1.225 ab
	30	10	103.520 fg	22.800 f	79.113 c	2.249 d
	30	20	68.866 bc	6.282 bcd	65.434 b	1.909 cd
	15	0	55.611 ab	4.553 b	47.542 a	1.475 bc
	15	5	92.701 defg	5.472 bc	183.827 f	1.969 cd
	15	10	99.003 efg	22.139 f	93.114 e	5.956 f
	15	20	76.250 cd	10.489 de	67.038 b	1.644 bc
½	30	0	71.266 bcd	4.383 ab	43.148 a	1.134 ab
	30	5	97.454 efg	6.549 bcd	67.303 b	1.876 cd
	30	10	108.787 g	6.744 bcd	67.837 b	1.309 b
	30	20	75.748 cd	16.099 e	70.117 bc	1.501 c
	15	0	64.735 abc	3.223 a	40.828 a	0.945 a
	15	5	86.437 def	12.207 de	60.902 b	2.778 e
	15	10	78.900 de	11.732 de	57.592 b	1.350 b
	15	20	73.423 cd	9.898 cde	70.913 c	3.066 e
3-wayANOVA						
Strength MS (A)			0.000***	0.001**	0.002**	0.000***
Sucrose level (B)			0.000***	0.000***	0.001**	0.000***
Mannitol concentration (C)			0.797 ns	0.000***	0.280 ns	0.000***
(A)*(B)			0.056 ns	0.157 ns	0.261 ns	0.482 ns
(A)*(C)			0.000***	0.002**	0.084 ns	0.000***
(B)*(C)			0.829 ns	0.000***	0.261 ns	0.000***
(A)*(B)*(C)			0.317 ns	0.010**	0.129 ns	0.000***
Rootstock: Gisela 6						
full	30	0	25.578 ab	2.801 ab	38.320 abcd	1.385 a
	30	5	28.264 abc	2.619 ab	66.995 fg	2.173 abc
	30	10	25.941 ab	7.865 ef	50.800 bcdef	4.255 d
	30	20	23.942 ab	4.729 cde	34.019 abc	1.669 abc
	15	0	38.220 cde	6.288 de	39.108 abcde	1.476 ab
	15	5	29.819 abcd	10.255 f	57.248 ef	2.375 c
	15	10	35.332 bcde	10.904 f	52.477 cdef	5.924 e
	15	20	25.840 ab	3.716 ab	33.026 ab	1.886 abc
½	30	0	39.895 de	3.547 ab	39.673 abcde	1.392 a
	30	5	22.508 a	5.651 cde	39.950 abcde	2.331 bc
	30	10	45.146 e	3.333 ab	76.061 g	1.729 abc
	30	20	28.284 abc	3.099 ab	53.789 def	1.924 abc
	15	0	34.039 abcd	2.398 a	40.902 abcde	1.665 abc
	15	5	32.121 abcd	5.761 cde	26.769 a	1.483 ab
	15	10	30.687 abcd	2.729 ab	59.968 fg	1.950 abc
	15	20	30.546 abcd	3.742 cd	41.592 abcde	1.995 abc
3-wayANOVA						
Strength MS (A)			0.001**	0.000***	0.216 ns	0.000***
Sucrose level (B)			0.391 ns	0.033*	0.000***	0.000***
Mannitol concentration (C)			0.003**	0.003**	0.010**	0.000***
(A)*(B)			0.196 ns	0.003**	0.369 ns	0.000***
(A)*(C)			0.012*	0.046*	0.781 ns	0.000***
(B)*(C)			0.493 ns	0.044*	0.037*	0.000***
(A)*(B)*(C)			0.581 ns	0.015*	0.283 ns	0.000***

It is possible that at high sugar concentration, rooting reduces the ability of buds to multiply by diverting most nutrients to root formation rather than to bud formation to overcome the expected water stress under these higher concentrations.

In the absence of mannitol from the culture medium, higher rooting percentages were recorded for CAB-6P explants with 30 g/l sucrose in half MS or with 15 g/l sucrose in full MS medium. Khan *et al.* (1999) found that the increase in sucrose concentration (from 10 to 30 g/l) was positively correlated with the rooting percentage and root number in *Syzygium alternifolium*, however, sucrose concentrations higher than 40 g/l inhibited overall rooting response. On the other hand, in Gisela 6, the rhizogenetic capacity of the explants was greater with 30 g/l sucrose in full MS or 15 g/l sucrose in half MS medium. In *Eucalyptus globules* and *Eucalyptus saligna*, increase of sucrose level from 30 to 60 g/l did not influence root number and rooting percentage considerably (da Rocha Corrêa *et al.* 2005).

Root elongation of both CAB-6P and Gisela 6 microcuttings was diminished when the strength of the medium in inorganics was reduced by half. The promotory effect of mineral concentration of the culture medium on rooting can be attributed to the participation of inorganic ions in processes regulating hormonal balance (Amzallag et al., 1992). The favourable effect of a diluted mineral solution on rooting can be explained by the reduction of nitrogen concentration (Driver and Suttle 1987). Dimassi-Theriou (1995) reported that reducing mineral concentration of the MS medium to half the normal value increased rooting percentage and stimulated root elongation of the GF 677 rootstock *in vitro*. Ruzic et al. (1984) have proposed the use of MS medium at half of normal strength for rooting improvement of the GF 677 rootstock shoots. Moncousin (1988) suggested that the dilution of salt concentration to half prepare the plants in the tubes for better adaptation to the acclimatisation medium, while shoot growth and leaf size were increased. Reducing mineral concentration of MS medium to half the normal value increased the rooting percentage, root number and root elongation of PR 204/84 (*Prunus persica* x *P. amygdalus*) (Fotopoulos and Sotiropoulos 2005). Root length of Gisela 6 explants was increased along with an increase in sucrose level (15 to 30 g/l) in full MS medium. Our findings are in line with those obtained in *Hypericum perforatum* (Cui *et al.* 2010), in *E. globules* and *E. saligna* (da Rocha Corrêa *et al.* 2005) and *S. alternifolium* (Khan *et al.* 1999). Similarly, El-Karzaz *et al.* (1997) found that in mulberry (*Morus alba* L.) plants root formation on *in vitro* shoots was most extensive on MS medium supplemented with 30 g/l sucrose.

In Gisela 6 explants grown on half MS medium, the increase in sucrose level from 15 to 30 g/l led to a significant decrease in root fresh weight. In *Hyoscyamus niger* L., 15 or 30 g/l sucrose produced good root biomass while higher concentrations had a negative effect (Hong *et al.* 2010). Sucrose generally exerts osmotic pressure and influences productivity of plants as a carbon source. In CAB-6P, on the other hand, non substantial fluctuations were observed in root fresh weight due to alterations in the MS medium regarding its sucrose and

inorganics levels. Our results in Gisela 6 are in tune with Kevers *et al.* (1999), who reported 30 g/l sucrose to be optimum for root biomass production in *Panax ginseng* and *P. quinquefolium*.

In CAB-6P explants, the higher sucrose level (30 g/l) and medium strength (full MS) promoted better shoot length and shoot fresh weight whereas in Gisela 6, 15 g/l sucrose + half MS gave better results. In micropropagated potato plantlets (Mohamed and Alsadon 2010) and in date palm (*Phoenix dactylifera* L.) cv. Khanezi (Al-Khateeb, 2001) increasing sucrose concentrations significantly increased plantlet height and shoot fresh weight. In CAB-6P, the supplementation of the full MS medium containing 30 g/l sucrose with 5 g/l mannitol positively influenced shoot length. On the other hand, Fortes and Scherwinski-Pereira (2001) in potato, Moges *et al.* (2003) in chrysanthemum and Shibli *et al.* (1999) in bitter almond found that mannnitol caused a reduction in shoot growth compared to sucrose. In olive (*Olea europaea* L.), however, mannitol gave higher shoot length than sucrose (García *et al.* 2002).

In CAB-6P, callus fresh weight was greatest in the half MS medium supplemented with 30 g/l sucrose and 5 g/l mannitol. Mannitol increased callus fresh weight when added in the full MS medium with 15 g/l sucrose. In *P. dactylifera* leaf explants cultured *in vitro,* callus fresh weight was increased with sucrose concentration up to 0.1 M but then declined (Asemota *et al.* 2007). In Gisela 6, half MS supplemented with 15 g/l sucrose (mannitol-free) exhibited greater callus fresh weight. Similarly, in strawberry when sucrose concentration was raised from 30 to 90 g/l callus fresh weight was decreased (Gerdakaneh *et al.* 2010). According to Gerdakaneh *et al.* (2009), low sucrose concentration is much more effective than high levels on callus fresh weight as the rise of sucrose concentration decreases callus fresh weight and this capacity could be attributed to an osmotic effect. In CAB-6P, the increase in sucrose concentration from 15 to 30 g/l led to lower callus induction frequencies. Similar findings were reported for Gisela 6 where callus induction frequency was greatest in the simultaneous effect half MS + 30 g/l sucrose + 5 g/l mannitol. In the apple rootstock Jork 9, sucrose promoted callus formation (Pawlicki and Welander 1995).In CAB-6P, mannitol led to depleted chlorophyll, carotenoid and porphyrin levels in half MS medium regardless sucrose content, except for the half MS + 30 g/l sucrose + 5 g/l mannitol combination treatment where an increase in total chlorophyll content was observed. In accordance, mannitol decreased chlorophyll and carotenoid content of sugarcane vitroplants (Cha-Um and Kirdmanee 2008). In Gisela 6, mannitol decreased carotenoid content when explants treated with 15 g/l sucrose (full MS). A possible explanation for this decrease according to Vitova *et al.* (2002) is that mannitol creates an osmotic stress which strongly inhibits the plant cell, tissue and organ growth mainly by impairing the gain of photoassimilates e.g. by inducing stomata closure or lowering the activity of photosynthetic enzymes. In Gisela 6, on the other hand, fifty percent decrease in both macro- and microelements concentration of MS medium supplemented with 15 g/l sucrose raised chlorophyll content. Sucrose linearly increases the level of reducing sugars, starch, and total chlorophyll in citrus plantlets (Hazarika *et al.*

2000). In CAB-6P, higher chlorophyll levels were recorded in half MS medium supplemented with 15 g/l sucrose or 30 g/l sucrose + 5 g/l mannitol compared to the full MS one. Different results were reported by Mohamed and Alsadon (2010) and Serret *et al.* (2001) who observed a positive correlation between chlorophyll content and sucrose concentration. The higher chlorophyll content observed in the rooting phase in plants growing at lower sucrose concentrations such as in Gisela 6 cherry rootstock of our study may indicate that such plants could present a higher photosynthetic capacity, facilitating the acclimatization.

In CAB-6P, mannitol resulted in elevated carbohydrate and proline levels in both leaves and roots regardless sucrose level (15 or 30 g/l) and concentration of the MS medium in inorganics (full or half). Accordingly, mannitol considerably augmented endogenous proline of micropropagated sugarcane (Cha-Um and Kirdmanee 2008). Vitova *et al.* (2002) states that under high mannitol concentration, osmotic stress causes restriction of mannitol utilization and lowering availability of energy and carbon source. In Gisela 6, mannitol increased leaf proline content of leaves grown in half MS medium with 15 g/l sucrose, whereas in roots mannitol at 10 g/l raised both proline (full MS) and carbohydrate content (half MS) irrespective sucrose level. According to Ahmad *et al.* (2007) sugars are perceived by cells as chemical signals *in vitro* with very high concentration acting as stressing agents.

Proline accumulation in stressed plants is one of the vital compatible solutes to function in cellular osmotic adjustment and scavenge detoxify oxidants (Seki *et al.* 2007) and its degradation can provide carbon, nitrogen and energy source after stress (Hare *et al.* 1999). On the contrary, the increase in sucrose level from 15 to 30 g/l led to depleted proline levels in Gisela 6 leaves and CAB-6P roots (full MS) indicating mechanism of osmoregulation and osmotic adjustment located only in leaves for Gisela 6 and only in roots for CAB-6P. The carbohydrate content of Gisela 6 leaves was higher in half MS medium supplemented with 30 g/l sucrose. Sucrose may facilitate growth and development due to its impact on the adjustment of cell osmolarity as reported by Khuri and Moorby (1995). In CAB-6P roots, the increase in sucrose level from 15 to 30 g/l resulted in depleted proline levels. Our findings are in disagreement with those presented in micropropagated potato plantlets (*Solanum tuberosum* L. cv Norland) (Badr *et al.* 2011) and in strawberry (Gerdakaneh *et al.* 2010) where the amount of free proline was increased as a response to the increase in sucrose concentration. According to Badr *et al.* (2011), the presence of sucrose in the culture medium causes an osmotic stress which leads to the build up of compatible solutes, such as proline, sugars and sugar alcohols in plantlets allowing them to absorb water under these conditions.

CONCLUSIONS

It seems that mannitol concentration, sucrose level and medium strength in inorganics are involved in photosynthetic apparatus, influencing leaf chlorophyll, carotenoid or porphyrin content and participating in carbohydrate biosynthesis and metabolism as well as in proline accumulation in both leaves and roots. It is

obvious that each cherry rootstock has its own specific requirements in macro- and micronutrients, in sucrose as the main carbon and energy source and in mannitol as a supplement, all in combination for optimum rooting and shoot growth performance under in vitro conditions.

Therefore, the different responses between the two cherry rootstocks to rooting, vegetative and callusing traits as well as to various biochemical parameters are genotype-dependent.

ACKNOWLEDGEMENTS

We would like to express our sincere gratitude to Fitotechniki Plant Tissue Culture Laboratory - Xylogiannis Bros Co., Arta, Greece and Angelos Xylogiannis for kindly providing the CAB-6P (P. cerasus L.) and Gisela 6 (P. cerasus x P. canescens) vitroplants; also our thanks to Sofia Kuti and Vasiliki Tsakiridou for technical assistance.

The authors gratefully acknowledge the financial support of the Aristotle University of Thessaloniki.

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