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In vitro Multiplication and Growth of Vegetative Rootstocks for Sweet Cherry as Affected by Dynamics of Macroelement Uptake from the Medium

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Abstract: The paper presents the relation between different macroelement concentrations in the medium, their uptake from the medium and multiplication of two vegetative sweet cherry rootstocks, Inmil GM 9 and Camil GM 79. Cultures were grown on Murashige and Skoog (MS) medium, on MS medium with double-strength, half-strength and 1/4 strength macro salts with mg l^{-1}: BA, 1; NAA, 0.1; and GA_3, 0.1. Control medium contained only deionized water and agar. All the analyses and multiplication parameters were monitored on day 0, day 20 and day 40 of culturing. The genotype Inmil GM 9 showed the best growth and development on MS, whereas genotype Camil GM 79 performed best on MS and MS 1/2 media. However, macroelements were mostly taken up from MS 1/2 and MS 1/4 media. On MS medium, which produced the best multiplication and growth in these genotypes, the highest uptake of N and P were recorded.

Key words: vegetative sweet cherry rootstocks, macroelements, uptake dynamics, growth, multiplication, *in vitro*

Introduction

Many factors affecting the *in vitro* growth of isolated cells, tissues and organs are similar to those limiting *in vivo* plant growth. Of the factors which influence numerous processes in *in vitro* environment, mineral nutrition and the process of the uptake of mineral elements and plant growth have been least studied so far (Leifert et al., 1995).

In vitro plant growth is mostly affected by the medium composition. To date, no universal medium for *in vitro* plant culture has been developed. Namely, plant species and cultivars are genetically specific concerning the different medium components, which includes not only organic substances, but mineral elements as well (Sarić et al., 1995).

In vitro plant growth is known to depend on mineral elements and organic media components, due to very low photosynthesis intensity and small leaf surface. Therefore, the choice of mineral and organic components of the medium is of particular importance for the success of this method (Lumsden et al., 1990).

Many tissue culture media are based on Murashige and Skoog medium composition (Murashige and Skoog, 1962), which was designed for cell cultures of *Nicotiana tabacum* and used in about 25% papers published on cell and tissue culture, as well as in over 50% of all micropropagated plants (Leifert et al., 1995). However, the studies carried out so far show the mineral composition of many media to be deficient or sufficient in some macroelements, which results in abnormal growth, hyperhydricity and many other undesirable effects in *in vitro* grown plants (Ružić, 1998).

The present paper aimed at assessing multiplication and growth of vegetative rootstocks for sweet cherry as affected by the dynamics of macroelement uptake from the medium.

Materials and method

Plant material. Two currently used low-vigorous rootstocks for sweet cherry, Inmil GM 9 (IN), interspecies hybrid *Prunus incisa* x *Prunus serrula*, and Camil GM 79 (CA), a clone of *Prunus canescens*, both developed at the Station for Fruit and Vegetable C.R.A.,Gembloux, Belgium,,were used as plant material.

The shoots of these two rootstocks had been multiplied *in vitro* for a year preceding trial establishment. In order to avoid the effect of the residues from the previous medium, prior to the placement on the appropriate media the shoots were subcultured once on them, so that the morphometric measurements and samples for chemical analyses were taken from the second subculture. Twenty-five culture vessels (100 ml Erlenmeyer flasks) x 5 uniform shoots x 2 replications were set per each treatment for day 20, and the same quantity for day 40, totalling 500 shoots per treatment x 5 media = 2,500 shoots per genotype.

Media. The cultures were grown on Murashige and Skoog medium (MS), on MS with double-strength macro salts, and on MS with macro salts reduced to 1/2 (MS 1/2) and 1/4 (MS 1/4). The control medium contained deionized water and agar. Prior to autoclaving, the pH value of all the media was adjusted to 5.75 with 0.1 N KOH. The media were sterilized in an autoclave for 20 minutes at 120°C. All the media contained agar at 7 g l^{-1} and sucrose, 20 g l^{-1}. The plant growth regulators used in all the treatments, except in the control, are as follows: 6-benzyladenine (BA), 1.0 mg l^{-1}; α-naphtylacetic acid (NAA), 0.1 mg l^{-1}, and gibberellic acid, GA_3, 0.1 mg l^{-1}.

Multiplication parameters. The studied parameters were measured on day 0 (in the medium - after autoclaving, in the explants - on the day of placing in culture), on day 20 and day 40 of culture.

Multiplication parameters were determined using standard morphometry. The shoots smaller than 0.5 cm were not taken into consideration.

Chemical analyses of the medium. After they had been taken out from the culture vessels, the media were mixed from a number of these vessels in one treatment and such a representative sample (in three replications per treatment) was frozen and kept until the analysis was performed.

Upon defrosting, 30 g of the medium was taken and held in water bath for 2 hours in porcelain vessels, then dried for 10-15 minutes at 105°C in the drier. Degradation of organic matter from the medium was carried out by dry digestion in the oven at 550°C for 48 hours. The residues of digestion were removed by heating vessels containing ash and 5 ml 3 N HCl up to boiling. The solution was then quantitatively filtered, and thus the stock or original solution from which the macroelements were determined, was prepared.

Potassium, calcium and magnesium were determined by atomic absorption spectrophotometry (AAS) - Pye Unicam and phosphorus by spectrophotometry - PU 8740 UV/VIS using the colorimetric vanadomolybdate method. Nitrogen was determined according to Kjeldahl procedure on Tecator-Kjeltec System 1003.

The samples were analyzed on day 0, day 20 and day 40 of culture. Thus, the total uptake of the elements by plants was calculated from that difference.

Culture conditions. The cultures were grown under 16/8 h photoperiod, light/dark with the light intensity of 8.83 Wm^{-1} on the culture surfaces provided by white fluorescent tubes 40 W, 6,500°K in strength. The temperature for growth in the chamber averaged 25° ± 1° C.

The data were statistically processed by the analysis of variance (ANOVA) and F-test, as well as by individual Duncan's Multiple Range Test.

Results

The genotype Inmil showed the best growth, as well as other multiplication parameters monitored, on MS medium (Tab. 1). The shoots on this medium had strong, long stems, compact, yellow-green or dark callus of firm consistency, and large, dark green leaves with long petioles (Fig. 1a). The MS 2x medium also produced strong and long stems, but with poorer multiplication. The shoots of this genotype had shortened growth habit on MS 1/2 medium, with large callus mass at the base, of soft consistency, covering a part of the stem, with small, curled and narrow leaves. The MS 1/4 medium produced a large mass of soft, pink callus at the shoot base of this genotype, which remained in the medium at removing the cultures. The stem was shortened and pervaded with callus, and leaves were narrow on long pink petioles and the occurrence of leaf tip yellowing (Fig. 1b). Shoot length on this medium was even smaller compared to that grown on control medium. Control medium gave no multiplication, but

produced wavy, thin, long, pink roots laterally from the shoot base, with white tips (Fig. 1c). They averaged about 5.5 cm up to day 40 of culture (Tab. 3).

Tab. 1. Multiplication parameters of Inmil GM 9 genotype

Media	Multiplication index		Length of axial shoot (cm)			Length of lateral shoot (cm)	
	20 *day*	40 *day*	0 *day*	20 *day*	40 *day*	20 *day*	40 *day*
MS 2x	1:1.44 ab	1:1.85 b	1.46 a	1.66 ab	2.14 a	0.68 a	0.88 a
MS	1:1.80 a	1:2.80 a	1.51 a	1.97 a	2.15 a	0.82 a	1.21 a
MS 1/2	1:1.20 b	1:1.44 bc	1.06 bc	1.19 bc	1.30 b	0.63 a	0.72 a
MS 1/4	1:1.18 b	1:1.32 bc	0.93 c	0.96 c	0.97 b	0.53 a	0.56 a
Control	1:1.00 b	1:1.00 c	1.16 b	1.18 bc	1.20 b	-	-

On day 20 of culture, the genotype Camil had the highest multiplication index on MS 1/2 and MS 1/4 media, whereas it produced the longest axial shoot on MS medium (Tab. 2). The medium MS 2x was unfavourable for growth and development of this genotype, since the shoots showed poor multiplication, hyperhydric stems and small leaves. With regard to both multiplication and shoot quality, the genotype Camil performed better on the media with reduced macro salts content as compared to Inmil. On control medium, Camil shoots had poor rooting, and short roots were formed (Tab. 3). The stem was brown, with light green leaves (Fig. 1d).

Tab. 2. Multiplication parameters of Camil GM 79 genotype

Media	Multiplication index		Length of axial shoot (cm)			Length of lateral shoot (cm)	
	20 *day*	40 *day*	0 *day*	20 *day*	40 *day*	20 *day*	40 *day*
MS 2x	1:1.19 b	1:1.58 c	0.96 b	1.15 a	1.19 b	0.56 c	0.58 a
MS	1:1.88 a	1:3.92 a	1.26 a	1.56 a	2.19 a	0.70 ab	0.97 a
MS 1/2	1:2.20 a	1:2.98 b	1.19 a	1.50 a	1.58 b	0.78 a	1.01 a
MS 1/4	1:2.20 a	1:3.70 a	0.96 b	1.10 a	1.14 b	0.62 bc	0.97 a
Control	1:1.00 b	1:1.00 c	0.95 b	1.00 a	1.01 b	-	-

Table 3. Rooting parameters in the control medium

Duration of sub-culture	Rooting %		Number of roots (Average ± SD)		Root length (cm) (Average ± SD)	
	Inmil	Camil	Inmil	Camil	Inmil	Camil
20 days	49.18	9.13	3.30 ± 0.59	1.81 ± 0.57	2.78 ± 0.13	0.90 ± 0.15
40 days	3.60	0	4.95 ± 0.63	1.14 ± 0.18	5.55 ± 0.15	1.56 ± 0.07

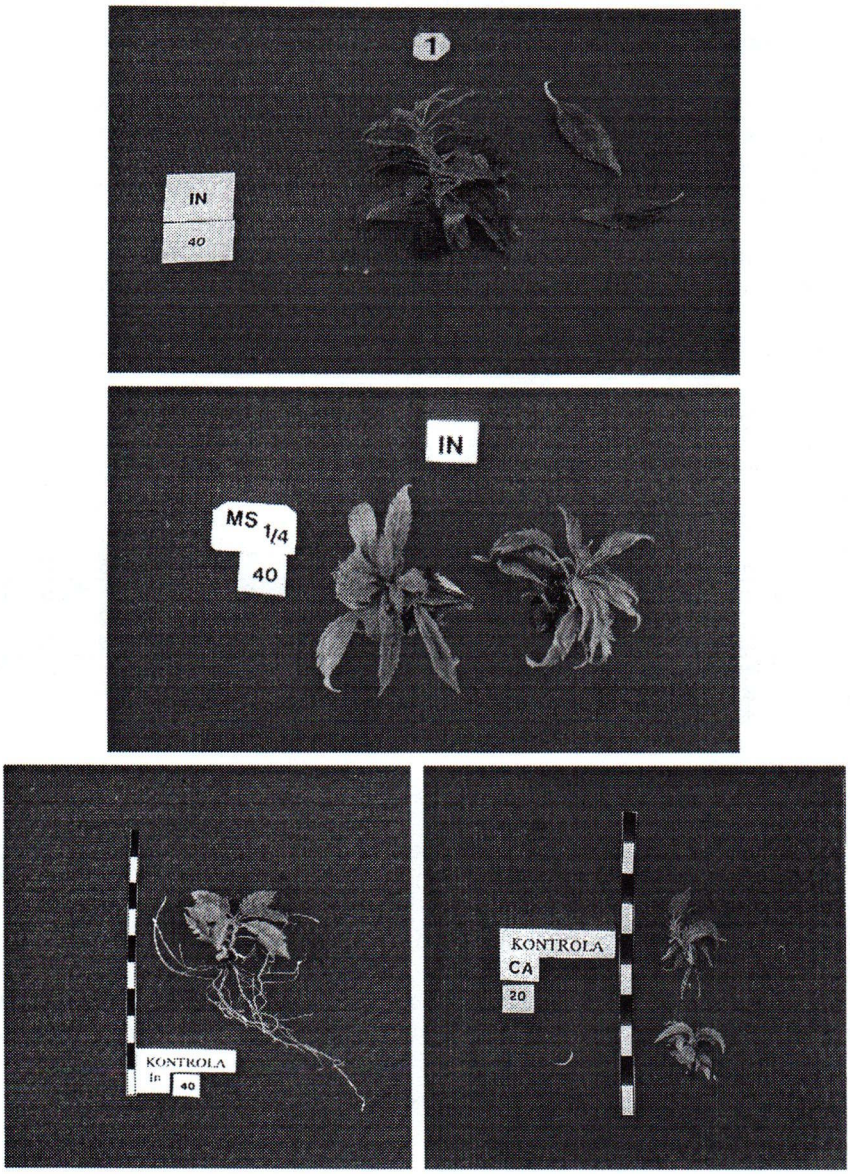

Fig. 1. Inmil shoots on MS medium (b) Inmil shoots MS 1/4 medium (c) rooted Inmil plantlet on control medium (d) Camil shoots on control medium

Duncan's Multiple Range Test revealed significant differences in multiplication parameters, i.e. in multiplication index and the length of axial shoot as follows: for Inmil on MS 2x and MS media on the one hand, and those grown on other media; in genotype Camil on MS 1/2 and MS, and for some parameters

also on MS 1/4 medium compared to the others. No significant differences in the length of lateral shoots in either genotypes were observed, particularly on day 40 of culture.

The shoots of Inmil, which had the best growth and multiplication on MS medium, took up the highest amount of N and P compared to other macroelements on day 20 - 45.9% and 42.9%, and 73.5% and 72.9% on day 40 of subculturing, respectively. The shoots of Camil also took up the highest levels of N and P from MS and MS 1/2 media, on which they had the best growth and multiplication; however, their uptake was superior from the media MS 1/2 (N-81.1%, P-50%) on day 20 and on day 40 of subculturing - 90.5% and 83.3%, respectively (Tab. 4).

Table 4. Dynamics of macroelement uptake from different media (in %)

Media/	N		P		K		Ca		Mg	
Genotypes	0 *day*= 5.65 *g*		0 day = 0.21 *g*		0 day = 4.74 *g*		0 day = 0.84 *g*		0 day = 0.26 *g*	
MS 2x	20 day	40 day	20 day	40 day	20 day	40 day	20 day	40 day	20 day	40 day
Inmil	24.8	57.4	52.4	81,9	19.2	30.5	10.0	24.1	4.0	24.0
Camil	20.4	36.3	42.9	65,9	12.9	12.6	14.1	20.0	18.2	9.1
MS	0 *day*= 2.91 *g*		0 day = 0.15 *g*		0 day = 2.14 *g*		0 day = 0.48 *g*		0 day = 0.15 *g*	
Inmil	45.9	73.5	42.9	72,9	22.1	46.2	34.0	68.1	33.3	53.3
Camil	46.6	79.9	50.0	68,8	29.9	42.5	20.8	37.5	37.5	62.5
MS 1/2	0 *day*= 1.54 *g*		0 day = 0.064 *g*		0 day = 0.71 *g*		0 day = 0.21 *g*		0 day = 0.09 *g*	
Inmil	80.3	95.2	83.7	83,3	11.6	78.3	28.6	76.2	50.0	80.0
Camil	81.1	90.5	50.0	83,3	44.9	66.7	28.6	61.9	44.4	77.8
MS 1/4	0 *day*= 0.76 *g*		0 day = 0.038 *g*		0 day = 0.49 *g*		0 day = 0.20 *g*		0 day = 0.08 *g*	
Inmil	80.5	93.9	29.7	54.1	67.4	89.8	40.0	80.0	33.3	73.3
Camil	59.5	79.8	23.7	50.0	60.0	88.0	35.0	55.0	21.1	73.7
Control	0 *day*= 0 *g*		0 day = 0 *g*		0 day = 0.06 *g*		0 day = 0.06 *g*		0 day = 0.01 *g*	
Inmil	-	-	-	-	-	25.0	-	-	-	-
Camil	-	-	-	-	50.0	33.3	28.57	42.9	50.0	50.0

Residues of other mineral elements on the media which produced the best growth and multiplication were considerably higher compared to N and P. However, the uptake of N, P and other macroelements in both genotypes was generally higher on media with reduced contents of macro salts, namely from MS 1/2 and MS 1/4.

Low levels of K, Ca and Mg were recorded in the control medium, which were mostly taken up by the genotype Camil, up to day 40 of culture.

Discussion

The shoots of both genotypes showed the best growth and multiplication on MS medium. The genotype Camil also had good multiplication and lateral shoot length on MS 1/2 medium. Since genotype multiplication and growth were best on MS medium, and the N and P uptake were also highest on this medium, growth and multiplication might be affected by the uptake of these macroelements. A limited addition of some essential ion is thought to be a possible factor of limited plant growth (Williams, 1995).

In the present paper, ion uptake was higher from the medium with reduced macroelement contents. It is known that the uptake of mineral elements

by plants depends on the levels of supply, but it is not always proportional to these levels (Kozai et al., 1995). However, the uptake of N and P was highest even on the media with reduced macroelement content, e.g. MS 1/2, compared to other macroelements.

The utilization of mineral elements usually has the opposite effect at a higher level of element supply and might be also affected by the element levels in plant tissue (Williams, 1993). The inhibitory effect of high levels of mineral elements in the medium on the uptake of minerals and plant growth can be overcome when the current supply is sufficiently high, i.e. if plants are grown at high pretreatment and subsequently transplanted to the medium containing high amounts of mineral elements, as was done in the present paper.

The uptake of mineral elements and plant growth are closely related. Both genotypes achieved best growth and development on the MS medium, with the highest uptake of N and P, too. Sakano et al. (1995) also reported a correlation between the uptake of P from the MS medium and cell growth in *Catharanthus roseus* (L.) G. Don.

Rapid uptake of certain elements might become a limiting factor to culture growth and development. In the present paper, although high amounts of N and P were taken up till the end of culturing, they did not represent the limiting factors to culture growth since no inhibition of growth and multiplication was observed until day 40 of culture. Despite the decrease of mineral elements in the medium, plants continued their growth since the internal deficit of elements had not occurred yet. Our cultures had an increased multiplication index and shoot length even on media with reduced amounts of macro salts on day 40 of culture compared to day 20 of subculturing. This does not imply, however, that the same would hold true for an interval of subculturing longer than 40 days.

Culture growth was observed on the control medium, too, particularly during the first subculture up to day 20, which was most probably affected by internal reserves of mineral elements in plants since towards day 40 of culture the growth was greatly slowed down.

As control medium contained only agar and water of high conductivity, it is supposed that the occurrence of K, Ca and Mg ions in small amounts in the medium on day 0 resulted from the presence of these elements in agar. Debergh (1983) found out that the impurities from agar induced significant differences in the element concentration in the medium, i.e. that the mineral element contents in gelling agents varied by the manufacturer. The agar used in the present paper, although it did not contain high amounts of available ions (Seingre et al., 1991), affected the element content in the control medium.

Both genotypes rooted on the control medium. The effect of culture transfer to the medium without growth regulators, the so-called 'hormone-free' medium for root induction (Cerović and Ružić, 1987; Snir, 1983), might be an explanation for root occurrence on the control medium in the present paper. The genotype Camil, which in contrast to the genotype Inmil rooted in low percent on this medium, showed also poor rooting on the media specific for rooting *in vitro* in the genus *Prunus* (Ružić, 1998). It still remains to be clarified, however, whether concentration reduction, i.e. omitting mineral elements from the

medium, or leaving out growth regulators and other organic substances, affected root formation on the control medium. The inhibitory effect of N on rooting (especially high concentration of ammonium ions) has not been determined yet, although plant growth and development reduction is thought to have an alternative direct effect on the expression of genes involved in the rooting process (Leifert et al., 1995). Identification of genes controlling the processes of shoot formation and rooting will define more precisely the regulatory effect of mineral elements.

Conclusion

At constant growth regulator concentrations, the multiplication and growth of these genotypes were mostly affected by N and P, both by the levels present in the medium, i.e. the concentration, and by the amount of uptake.

Higher concentrations of macroelements in MS medium did not have an inhibitory effect on culture growth and multiplication, and were almost optimum for multiplication and growth of these genotypes.

Other macroelements were mostly taken up from the medium with reduced amounts of macro salts, with unsatisfactory growth and multiplication, with the exception of MS 1/2 medium on which the genotype Camil had a good multiplication index and lateral shoot length.

Up to the end of culture period, neither growth nor multiplication have been suppressed although high levels of N and P were taken up both from MS and other media.

Different responses of these two genotypes to the mineral composition of the medium, i.e. on the reduced macroelement content in the medium, indicate the effect of a genetic factor on the mineral nutrition *in vitro*.

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MULTIPLIKACIJA I RASTENJE VEGETATIVNIH PODLOGA ZA TREŠNJU *IN VITRO* U ODNOSU NA DINAMIKU USVAJANJA MAKROELEMENATA IZ MEDIJUMA

- originalan naučni rad -

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Rezime

U radu je proučavan odnos između različite koncentracije makroelemenata u medijumu, njihovog usvajanja iz medijuma i multiplikacije dve vegetativne podloge za trešnju, Inmil GM 9 i Camil GM 79, *in vitro*. Kulture su gajene na Murashige i Skoog (1962) (MS) medijumu, na MS medijumu sa makro solima uvećanim 2x i smanjenim na 1/2 i 1/4 sa u mg l^{-1}: BA, 1; NAA, 0,1; i GA_3, 0,1. Kontrolni medijum sadržavao je samo vodu i agar. Sve analize i parametri multiplikacije su praćeni nultog, 20. i 40. dana gajenja. Najbolje rastenje i razviće genotip Inmil GM 9 je imao na MS, a genotip Camil GM 79 na MS i MS 1/2 medijumu, sa najvećim usvajanjem makroelemenata iz MS 1/2 i MS 1/4 medijuma. Na MS medijumu na kome je dobijena najbolja multiplikacija i rastenje ovih genotipova najviše su se usvojili N i P.