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Medium strength in inorganics and PVP concentration effects on cherry rootstocks *in vitro* rooting

V. Sarropoulou, K. Dimassi-Theriou, I. Therios

Laboratory of Pomology, Department of Horticulture, School of Agriculture, Aristotle University of Thessaloniki, Thessaloniki, Greece

Abstract

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The effects of two strength media (full and half) in inorganics and five polyvinylpyrrolidone (PVP) concentrations combined with 1 mg/l indole-3-butyric acid on the *in vitro* rooting of the CAB-6P, Gisela 6 and M × M 14 cherry rootstocks were investigated. For the CAB-6P, root number (5.80) was greatest by adding 5 g/l PVP to the half strength medium and 1 g/l PVP resulted in 80% rooting percentage. For Gisela 6, 1 g/l PVP in half strength medium led to the highest root number (6.30) as well as the highest rooting percentage (90.91%). On the contrary, root length was maximum (36.17 mm) in the full strength medium without adding PVP. In M × M 14, root number was maximum (8.33) in the PVP-free full strength medium, rooting percentage was 100% by adding 5 g/l PVP to either full or half strength medium and root length was greatest by incorporating 1 or 2.5 g/l PVP into the half strength medium. PVP seems to be a promising rooting agent in tissue culture systems.

Keywords: antioxidants; auxins; callus formation; plant tissue culture; rhizogenesis

The clonal cherry rootstock CAB-6P (*Prunus cerasus* L.) is a commercial cherry rootstock with limited practical use compared to *Prunus avium* L. and *Prunus mahaleb* (Dimassi-Theriou, Therios 2006). In general, sour cherry as a rootstock is reported to be winter-hardy, tolerant to wet soils but inconsistent compatibility with some sweet cherry cultivars. CAB-6P is a rootstock suitable for sweet and sour cherry varieties. Trees in the nursery are moderately vigorous and have an intermediate growth habit. The root system is moderately developed with good anchorage in the ground; tendency to suckering. It adapts well to heavy soils with low permeability, it is resistant to calcareous soils (9% active lime) as well as to nematodes and it is suitable for replanting.

Gisela 6 is a hybrid of *Prunus cerasus* × *Prunus canescens* and used as a semi-dwarf rootstock for all kinds of sweet cherry. It is also satisfactory in a wide range of soils, especially heavy soils (Andersen et al. 1999). The average and slow growth of this rootstock has a great value for the establishment and development of modern intensive cherry orchards. Trees grafted on this rootstock do not have any suckering problems (Long, Kaiser 2010). Producing Gisela 6 rootstock in large scale using conventional methods such as cuttings and layering in order to meet the growing internal demand

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for high quality, disease-free and uniform planting material seems to be difficult (Aka-Kacar et al. 2010). The scale and speed of the production of healthy plants can be enhanced using micropropagation techniques (Aka-Kacar et al. 2010).

The M × M 14 cherry rootstock is believed to be derived from a cross between *P. avium* cv. Mazzard and *P. mahaleb* L. Trials in France demonstrated that M × M 14 is semi-dwarf and improved the precocity of scion cultivars (Kappel, Lichou 1994). Tree size was 60–80% less than trees grafted on the standard cherry rootstock F12/1 (Kappel 1993). M × M 14 adapted well and resulted in enhanced productivity of cherry trees grafted on it. M × M 14 bears fruit by 4–5 years earlier than Mazzard F12/1 and has a high yield index (Edin et al. 1996). It is also reported that trees grafted on M × M rootstock series (mahaleb × mazzard) are smaller and exhibit higher yield efficiency than those grafted on mahaleb seedling rootstocks (Hrotkó et al. 1997). Plants grafted on M × M 14 rootstock are about 20% more vigorous than those grafted on CAB-6P and about 30% less vigorous than those grafted on sweet cherry seedlings (*Prunus avium*) (Dimassi-Theriou, Therios 2006).

In comparison with the traditional propagation (seeds, cuttings), the *in vitro* propagation procedure has several advantages. For the large-scale production of plants through *in vitro* techniques the important attributes are the quality, cost-effectiveness, maintenance of genetic fidelity and long-term storage (Mohan Jain, Häggman 2007). Micropropagation may be utilised in basic research, the production of virus-free planting material, the cryopreservation of endangered and elite woody species and in tree breeding (Mohan Jain, Häggman 2007).

Even though oxidation on the basis of mini-cuttings adversely affects the entire rhizogenic process, it can be minimised or even avoided by adopting some procedures such as using antioxidant substances, reducing mechanical damage or washing vegetative propagules under running tap water (Wendling 2002). Effects of antioxidants include inactivation of free radicals, complexometry of metabolic ions or reduction of peroxides in products unable to form free radicals (Araújo 2008). Antioxidants can potentially protect the natural plant rooting hormones from oxidation by enhancing rooting and increasing the tolerance of plants under greenhouse conditions (Lis-Balchin 1989). Their use is often reported in micropropagation whereas in the rooting of vegetative cuttings their use is very restricted (Fachinello et al. 1993). According to Teixeira (2001), PVP is an adsorbent compound that binds to phenols and therefore prevents oxidation.

The aim of the current research was to evaluate the effects of the exogenously applied PVP combined with two different strengths culture media in inorganics on *in vitro* rooting of CAB-6P, Gisela 6 and M × M 14 cherry rootstocks.

MATERIAL AND METHODS

Plant material and culture conditions. The experimental plant material was shoot tip explants from previous *in vitro* cultures (Fitotechniki Bros Co., Plant Tissue Culture Laboratory, Arta, Greece). These shoot tips were initially placed in solid MS culture medium (Murashige, Skoog 1962) supplemented with 1 mg/l 6-benzyladenine (BA), 0.01 mg/l indole-3-butyric acid (IBA) and 0.01 mg/l gibberellic acid (GA_3) for proliferation. In this way, a sufficient amount of *in vitro* mother stock shoots was obtained for further experiments. After 3 months of proliferation multiplied shoot tips were transferred on solid MS plant growth regulators (PGR) free medium for elongation. After 2 months of development in hormone-free MS medium shoot tips (1.5–2.5 cm long) were used as explants for the experiments. The initial material was certified as virus free. Two culture media different in their concentrations in mineral salts, full and half, were used. PVP was added at four concentrations (1, 2.5, 5 and 10 g/l) in combination with 1 mg/l IBA. The two PVP-free media (full and half strength in inorganics) containing only 1 mg/l IBA were treated as control treatments. The explants were grown in glass flat bottom test tubes (25 × 100 mm) containing 10 ml of MS medium. The culture medium was also supplemented with 30 g/l sucrose and 6 g/l agar (Bacto-Agar; Voigt Global Distribution Inc., Lawrence, USA). The pH of the culture medium was adjusted to 5.8 before adding agar and then the medium was sterilised by autoclaving at 121°C for 20 minutes. One explant was aseptically transferred to each test tube which was capped with aluminium foil. All the cultures were incubated in a growth room under controlled environmental conditions i.e. a 150 μmol/m^2/s light

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intensity provided by cool white fluorescent lamps (36W, Philips), a 16 h photoperiod and a 22 ± 1°C temperature. Mean root number per rooted explant, root length, root fresh mass (FM), rooting percentage, shoot length and shoot FM, callus FM and callus induction frequency were recorded after seven weeks of maintenance in the rooting media for CAB-6P, eight weeks for Gisela 6 and four weeks for M × M 14 rootstock in order to obtain full response.

Statistical analysis. The experimental layout was completely randomised and the data were analysed with ANOVA (Analysis of Variance) using the statistical package SPSS 17.0 (SPSS Inc, Chicago, USA). For each cherry rootstock, the experiment was a 2 × 5 factorial with two strength media in inorganics (full and half) and five PVP concentrations. The main effect of factors (strength medium, PVP concentration) and their interaction were determined by the General Linear Model (2-way ANOVA). The experiment was repeated twice and the reported data are the means. The experiment consisted of 10 treatments each one with a total of 20 replicates in the CAB-6P, 23 replicates in the Gisela 6 and 10 replicates in the M × M 14 rootstock, dependent on the available plant material. To establish significant differences among the treatments, the Duncan's multiple range test was used at $P \leq 0.05$ for mean comparison.

RESULTS

In CAB-6P, callus FM was maximum (0.070 g) in full strength medium PVP-free and 0.072 g when adding 2.5 g/l PVP (Table 1). Callus induction frequency was maximum (85%) in full strength medium when PVP-free or when supplemented with 1 g/l PVP. A significant reduction in shoot length was observed in half strength medium with 10 g/l PVP. Root number was significantly increased (5.1) by adding 10 g/l PVP to full strength medium. The rooting percentage was maximum (80%) in half strength medium when PVP-free or after adding 1 g/l PVP. Best rooting results in terms of root number (5.8) and root FM (0.075 g) were obtained in half strength medium with 5 g/l PVP. Neither medium strength in inorganics nor PVP concentration had an effect on root length or shoot FM.

In Gisela 6, 1 g/l PVP gave the same max. number of roots (6.3) in both full and half strength media (Table 1). The control treatment in full strength medium gave the max. root length (36.17 mm). The addition of 1 g/l PVP to full strength medium resulted in the highest root FM (0.080 g). Best results regarding the vegetative growth of the explants i.e. shoot length and shoot FM were recorded with 2.5 g/l PVP in full strength medium. The reduction of the full MS medium in inorganics led to the increase of the rooting percentage from 68.18% to 77.27%. The same increasing trend was observed when adding 1 g/l PVP to the half MS medium compared to the full MS one. Explants treated with 1 g/l PVP in the half strength medium gave the maximum rooting percentage (90.91%). The addition of 2.5 g/l PVP to full or half strength medium gave the max. callus FM (0.049 g) and callus induction frequency (72.73%), respectively. The greatest rooting percentage (90.91%) was recorded in half MS medium with 1 or 10 g/l PVP.

In M × M 14, root number (8.33) and root FM (0.198 g) were max. in full strength medium PVP-free (Table 1). The rooting percentage was highest (100%) with 5 g/l PVP in half strength medium. Root length and rooting percentage were doubled by decreasing the concentration of MS medium strength in mineral salts by half. Half strength medium with 1 or 2.5 g/l PVP resulted in the greatest root length (50.41–52.64 mm). PVP (1–10 g/l) in full strength medium increased rooting percentage from 30% to 70–100% but decreased root FM. In half strength medium, 2.5 and 5 g/l PVP augmented rooting percentage from 70% to 90% and 100%, respectively. Neither medium strength in inorganics nor PVP concentration affected shoot length and shoot FM as no significant changes were observed in these two vegetative growth parameters among treatments. Callus FW was greatest (0.088 g) in the full MS medium PVP-free. PVP (1–10 g/l) in full MS medium decreased callus FM whereas in the half MS one the highest PVP concentration of 10 g/l increased callus FM. Callus induction frequency was maximum (50%) with 5 g/l PVP in full strength medium. Callus induction frequencies in full strength media containing PVP (1–10 g/l) were substantially higher than those in half strength ones.

DISCUSSION

PVP, an antioxidant substance, affected *in vitro* callus induction, vegetative growth and rooting of

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Table 1. Effect of medium strength in inorganics and polyvinylpyrrolidone (PVP) concentration on cherry rootstocks rooting, vegetative and callus characteristics

MS strength in inorganics	PVP (g/l)	Root number/ rooted explant	Root length (mm)	Root fresh mass (g)	Rooting percentage (%)	Shoot length (mm)	Shoot fresh mass (g)	Callus fresh mass (g)	Callus induction frequency (%)
CAB-6P									
	0	2.79^{a}	29.93^{a}	0.050^{ab}	70^{d}	16.50^{b}	0.124^{ab}	0.070^{de}	85^{g}
	1	3.82^{abc}	26.45^{a}	0.050^{ab}	55^{c}	15.25^{ab}	0.115^{a}	0.065^{cde}	85^{g}
Full	2.5	3.50^{ab}	30.44^{a}	0.062^{abc}	70^{d}	17.00^{b}	0.154^{b}	0.072^{e}	60^{f}
	5	3.30^{ab}	25.19^{a}	0.043^{a}	52.63^{c}	15.26^{ab}	0.117^{a}	0.055^{bcde}	42.11^{c}
	10	5.10^{cd}	29.96^{a}	0.055^{abc}	50^{c}	15.50^{ab}	0.090^{a}	0.032^{a}	50^{e}
	0	4.13^{abc}	31.67^{a}	0.068^{bc}	80^{f}	15.50^{ab}	0.111^{a}	0.048^{bc}	45^{d}
	1	4.63^{bcd}	26.28^{a}	0.063^{abc}	80^{f}	15.25^{ab}	0.098^{a}	0.068^{de}	35^{b}
Half	2.5	3.93^{abc}	30.39^{a}	0.048^{ab}	75^{e}	15.75^{ab}	0.105^{a}	0.043^{ab}	30^{a}
	5	5.80^{d}	23.36^{a}	0.075^{c}	75^{e}	14.25^{ab}	0.112^{a}	0.054^{bcd}	30^{a}
	10	3.14^{ab}	27.37^{a}	0.046^{ab}	35^{a}	13.00^{a}	0.119^{a}	0.062^{cde}	30^{a}
2-way ANOVA									
Strength MS (A)		0.042^{ns}	0.733^{ns}	0.074^{ns}	0.000***	0.088^{ns}	0.116^{ns}	0.263^{ns}	0.000***
PVP (B)		0.175^{ns}	0.082^{ns}	0.735^{ns}	0.000***	0.253^{ns}	0.162^{ns}	0.011*	0.000***
A × B		0.000***	0.940^{ns}	0.008**	0.000***	0.841^{ns}	0.011*	0.000***	0.000***
Gisela 6									
	0	3.87^{ab}	36.17^{c}	0.052^{bc}	68.18^{d}	24.09^{bc}	0.181^{cd}	0.046^{cd}	59.09^{g}
	1	6.29^{c}	29.45^{abc}	0.080^{d}	77.27^{f}	25.00^{bc}	0.213^{d}	0.038^{bcd}	36.36^{b}
Full	2.5	3.54^{ab}	31.59^{bc}	0.044^{bc}	56.52^{c}	26.30^{c}	0.201^{d}	0.049^{d}	56.65^{f}
	5	5.20^{bc}	26.32^{ab}	0.044^{bc}	50.00^{b}	23.50^{abc}	0.158^{bc}	0.034^{abc}	55.00^{f}
	10	2.89^{a}	23.87^{a}	0.020^{a}	40.91^{a}	20.23^{ab}	0.134^{ab}	0.037^{abc}	54.55^{e}
	0	5.29^{bc}	28.37^{ab}	0.045^{bc}	77.27^{f}	22.27^{abc}	0.149^{abc}	0.039^{cd}	36.36^{b}
	1	6.30^{c}	32.10^{bc}	0.062^{cd}	90.91^{h}	20.91^{ab}	0.141^{abc}	0.026^{a}	45.45^{c}
Half	2.5	4.69^{abc}	31.09^{abc}	0.048^{bc}	72.73^{e}	19.09^{a}	0.105^{a}	0.027^{ab}	72.73^{h}
	5	4.28^{abc}	27.67^{ab}	0.038^{ab}	81.82^{g}	20.91^{ab}	0.130^{ab}	0.045^{cd}	50.00^{d}
	10	4.85^{abc}	26.37^{ab}	0.054^{bc}	90.91^{h}	22.95^{abc}	0.146^{abc}	0.039^{bcd}	31.82^{a}
2-way ANOVA									
Strength MS (A)		0.093^{ns}	0.758^{ns}	0.734^{ns}	0.000***	0.009**	0.000***	0.028*	0.000***
PVP (B)		0.000***	0.014*	0.000***	0.000***	0.906^{ns}	0.125^{ns}	0.135^{ns}	0.000***
A × B		0.617^{ns}	0.110^{ns}	0.011*	0.000***	0.026*	0.003**	0.001**	0.000***
M × M 14									
	0	8.33^{b}	25.72^{ab}	0.198^{d}	30^{a}	22.50^{a}	0.160^{a}	0.088^{f}	10^{b}
	1	5.50^{a}	29.10^{ab}	0.130^{c}	80^{d}	28.50^{a}	0.184^{a}	0.048^{de}	30^{d}
Full	2.5	6.00^{ab}	33.84^{ab}	0.133^{c}	90^{e}	25.50^{a}	0.179^{a}	0.059^{e}	40^{e}
	5	4.60^{a}	34.69^{ab}	0.092^{bc}	100^{f}	27.50^{a}	0.179^{a}	0.039^{cd}	50^{f}
	10	3.57^{a}	35.97^{b}	0.057^{ab}	70^{c}	24.00^{a}	0.124^{a}	0.021^{b}	20^{c}

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Table 1 to be continued

MS strength in inorganics	PVP (g/l)	Root number/ rooted explant	Root length (mm)	Root fresh mass (g)	Rooting percentage (%)	Shoot length (mm)	Shoot fresh mass (g)	Callus fresh mass (g)	Callus induction frequency (%)
M × M 14									
	0	6.14^{ab}	49.50^{c}	0.128^{c}	70^{c}	27.50^{a}	0.191^{a}	0.032^{bc}	10^{b}
	1	5.43^{a}	52.64^{c}	0.123^{c}	70^{c}	24.50^{a}	0.145^{a}	0.000^{a}	0^{a}
Half	2.5	6.11^{ab}	50.41^{c}	0.103^{bc}	90^{e}	30.00^{a}	0.147^{a}	0.022^{b}	20^{c}
	5	4.30^{a}	23.04^{ab}	0.049^{ab}	100^{f}	27.00^{a}	0.139^{a}	0.039^{cd}	30^{d}
	10	3.83^{a}	20.39^{a}	0.031^{a}	60^{b}	22.00^{a}	0.167^{a}	0.059^{e}	10^{b}
2-way ANOVA									
Strength MS (A)		0.382^{ns}	0.015*	0.005**	0.000***	0.762^{ns}	0.586^{ns}	0.000***	0.000***
PVP (B)		0.000***	0.005**	0.000***	0.000***	0.555^{ns}	0.718^{ns}	0.000***	0.000***
A × B		0.522^{ns}	0.000***	0.576^{ns}	0.000***	0.519^{ns}	0.131^{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 the Duncan's multiple range test at $P \leq 0.05$; significant effects at $P \leq 0.05$ (*), 0.01 (**), or 0.001 (***) according to 2-way ANOVA

CAB-6P, Gisela 6, and M × M 14 cherry explants in a dose-dependent manner. Furthermore, the three cherry rootstocks tested responded to medium strength in inorganics and PVP concentrations in different ways due to their different genotypes.

The addition of 1 g/l PVP to half strength medium increased the rooting percentage of CAB-6P and Gisela 6 explants to its maximum. In the M × M 14 rootstock, both full and half strength MS media supplemented with 5 g/l PVP resulted in 100% rooting percentage. In contrast, in mini-cuttings of three clones of *Eucalyptus urophylla* × *Eucalyptus grandis,* PVP was found to have the opposite effect on rooting percentage (de Melo et al. 2011). Similarly, in marula tree (*Sclerocarya birrea* subsp. *caffra)*, 0.8 g/l PVP in combination with 1 mg/l IBA gave the max. rooting percentage (68%) (Mollel, Goyvaerts 2012). In Virginia pine (*Pinus virginiana* Mill.), shoot rooting increased by 19% after adding 5 g/l polyvinylpolypyrrolidone (PVPP) (Tang et al. 2004).

Although in half strength medium Gisela 6 and CAB-6P microshoots formed more roots with 1 and 5 g/l PVP, respectively; in full strength medium M × M 14 shoot tips responded better in the absence of PVP. In the dwarf apple rootstock P 60, the half strength MS medium resulted in higher root number and root length than the full strength one (Orlikowska 1992). Neither strength medium nor PVP concentration affected root length of CAB-6P explants. On the other hand, in Gisela 6 microcuttings the half strength medium in inorganics supplemented with 1 or 2.5 g/l PVP and in M × M 14 rootstock the full strength medium without PVP both led to max. root length. In the apple cvs Delicious and Starkspur PVP had absolutely no effect on root number or root length and even though it decreased rooting percentage in the induction phase it had a stimulatory effect during the initiation and elongation phase (Standardi, Romani 1990). In Magnolia cv. Ann cultures PVP did not influence root number remarkably (Parris 2012).

In other plant species, PVP also had a stimulating effect on the rooting process. More specifically, in cotton (*Gossypium hirsutum* L. cv. SVPR2) plants PVP (17–20 mg/l) increased root number, root length and rooting percentage (Ganesan, Jayabalan 2006). In *Betula platyphylla* var. *japonica*, 100 mg/l PVP increased root length (Vaario et al. 1995). *Taxus mairei* embryos cultured for six weeks in half MS medium with 0.8 g/l PVP enhanced root elongation and gave 90% rooting percentage (Chang, Yang 1996). In oak (*Quercus robur* L.) microcuttings, 10 g/l PVP considerably enhanced root length and slightly increased the rooting percentage from 81.5 to 86.6% (Malá et al. 1999).

The concentration of inorganic salts plays an important role in root induction as the reduction of the original concentration of MS medium salts by

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75% stimulated root formation in date palm tissue culture (Ibrahim 1999). The mineral concentration of the culture medium affects rooting characteristics and for this reason some researchers have proposed its reduction to half normal strength for rooting improvement (Dimassi-Theriou, Economou 1993) since inorganic ions participate in processes regulating hormonal balances (Amzallag et al. 1992). The favourable effect of a diluted mineral solution on rooting can be explained by the reduction in nitrogen concentration (Driver, Suttle 1987). Dimassi-Theriou (1995) reported that by reducing mineral concentration of the MS medium to half resulted in the increase of rooting percentage and root elongation of the GF 677 peach rootstock *in vitro*. Ruzic et al. (1984) proposed the use of half strength MS medium for rooting improvement of the GF 677 rootstock shoots. Fouad et al. (1997) reported that a half strength medium supplemented with different concentrations of auxin resulted in high rooting percentages of the *Prunus* rootstocks Nemaguard and Meet-Ghamre. Reducing mineral concentration of MS medium to half increased the rooting percentage of PR 204/84 (*Prunus persica* × *Prunus amygdalus*) shoots when IBA concentration was 0.5 mg/l, and the root number when IBA concentrations were 0.5 and 1 mg/l. Root elongation was stimulated in all IBA concentrations in both full and half strength media, even though the differences among the treatments were subtle (Fotopoulos, Sotiropoulos 2005). In *Eucalyptus sideroxylon* seedlings after a 4-week treatment with 1 mg/l IBA or α-naphaleneacetic acid (α-NAA), rooting percentage and root length were lower in full strength MS than in the half one (Cheng et al. 1992).

In all three cherry rootstocks studied, no significant changes were observed in terms of shoot length among treatments. Similarly, PVP (1 g/l) did not modify substantially shoot length of Magnolia cv. Ann cultures in all media tested (MS, half-strength MS, WPM, Blaydes, DKW) (Parris 2012). In CAB-6P and Gisela 6 rootstocks, 2.5 g/l PVP in full strength medium slightly increased callus FM whereas in M × M 14 rootstock PVP had a negative effect. Callus induction frequency was promoted by adding PVP to the full strength medium in CAB-6P and M × M 14 explants but in Gisela 6 the same result was obtained when PVP was incorporated into the half strength medium. Similar results were obtained in cotton (*Gossypium hirsutum* L. cv. SVPR2) plants where 21–25 mg/l PVP led to callus formation (Ganesan, Jayabalan 2006). Our findings are also in agreement with those reported by Reustle and Natter (1994), where the formation of microcalli from grapevine protoplasts was remarkably improved when adding PVP at Day 0 or Day 7 of cultivation. Inclusion of 6 g/l PVPP in the MS culture medium further improved callus induction of "Tifway" Bermudagrass (*Cynodon transvaalensis* Burtt-Davy × *C. dactylon* (L.) Pers) young inflorescence culture (Qu, Chaudhury 2001).

In conclusion, the rhizogenetic capacity of CAB-6P and Gisela 6 rootstock explants was enhanced by adding PVP and reducing the concentration of MS medium in macronutrients and micronutrients to half. In M × M 14 rootstock, PVP along with a reduction in mineral salt concentration stimulated root length whereas root number and callus induction frequency were affected negatively. It is clear that the differences among the three cherry rootstocks regarding their response to the rooting process are genotype-dependent.

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Corresponding author:

Dr. Virginia Sarropoulou, Aristotle University of Thessaloniki, School of Agriculture, Department of Horticulture, Laboratory of Pomology, 54124, Thessaloniki, Greece
phone: + 30 2310 908 377; e-mail: vsarrop@gmail.com