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In vitro propagation of plum rootstocks

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Abstract. To determine the effect of successive subculturing on multiplication capacity of shoots, three rootstocks for plum, i.e. Cherry plum (*Prunus cerasifera* Ehrh.), 'Docera 6' (*P. domestica* × *P. cerasifera*) and 'Dospina 235' (*P. domestica* × *P. spinosa*) were repeatedly subcultured for five subcultures on Murashige & Skoog (MS) medium of unchanged plant growth regulator composition. Monitoring of the shoot formation capacity revealed constant increase in multiplication index and length of axial and lateral shoots in Cherry plum during each consecutive multiplication cycle. As for 'Docera' and 'Dospina', significan increase in multiplication index and length of axial shoots was observed in the third subculture. After that their values gradually decreased to the fifth subculture, but remained considerabely higher in comparison with the values in the first two subcultures. This study has confirmed that response of *in vitro* cultures to constant subculturing is genotype dependant. Considering that the decline in multiplication index was already observed after third subculture, it is neccesary to determine when cytokinin type and concentration should be reduced, or the hormon-free medium should be deployed to delay the decrease. MS medium with mineral salts reduced to ½-strength and organic complex unchanged was used in rooting stage. The highest capacity for *in vitro* rooting was observed in Cherry plum (100%), followed by 'Docera' (91.7%), while the lowest was in 'Dospina' (51.9%). Therefore, an additional optimization of this stage by testing other types and concentration of auxin should be done in order to achieve higher rooting percentage in 'Dospina'.

Key words: Cherry plum, 'Docera', 'Dospina', *in vitro*, micropropagation

Introduction

Temperate fruit tree rootstocks are traditionally propagated using vegetative methods (time-consuming and labor intensive process), or by seeds which does not guarantee uniformity of material obtained in such manner. The application of micropropagation for propagation of fruit rootstocks started in the mid-1970s, and a considerable number of improved protocols were developed ever since (cited in Vujović et al., 2012).

In plum rootstocks this method has been successfully applied with different clones of Cherry plum (*Prunus cerasifera* Ehrh.) such as Mr.S. 2/5 (Fortuna et al., 1996), Myrobalan 29C (Shabani et al., 2015), Dzanka 4 (Nacheva et al., 2002), as well as with vegetative rootstocks – Jaspi (Vujović et al., 2012; Mahajan et al., 2017) and St. Julien A (Garoosi et al., 2018). Most of the stated resaerch were focused on the influence of the type and/or concentration of plant growth regulators (PGRs) on multiplication and rooting capacity of *in vitro* shoots. However, the effect of repeated subculturing on shoot growth and multiplication was less considered in literature (Vujović et al., 2012). A decrease in multiplication capacity during long-term growth of shoots on medium of constant PGR composition was reported in pineapple (Hamad & Taha, 2008) as well as in vegetative rootstocks belonging to the *Prunus* genera (Vujović et al., 2012). However, the point of decline is highly dependent on tissue culture

in vitro conditions (PGR composition of medium, subculture period etc.). Contrary, in dwarf raspberry (*Rubus pubescens* Raf.), Debnath (2004) noticed that both shoot multiplication index and shoot length increased during subculturing up to the third subculture, and then remained constant.

This paper presents protocol for *in vitro* propagation of three plum rootstocks: Cherry plum, 'Dospina 235' and 'Docera 6'. Cherry plum, also known as Myrobalan plum, is one of the most widely grown fruit cultures in the native population of fruit species in Serbia (Nikolić & Rakonjac, 2007). Myrobalan seedlings are traditionally and commonly used as rootstock for plums. 'Dospina 235' and 'Docera 6' rootstocks were released from plum rootstock breeding program established at Technische Universität München. The aim of this program was to develop semidwarfing and dwarfing rootstocks with hypersensitivity resistance to *Plum pox virus* (PPV) (Neumüller et al., 2013). In this study special attention was devoted to the study of consecutive multiplication cycles on shoot formation capacity and growth of *in vitro* shoots repatedly subcultured on the medium of the constant PGR composition which can significantly affect the efficiency of micropropagation.

Material and Methods

Three plum rootstocks i.e. Cherry plum (*Prunus cerasifera* Ehrh.), 'Docera 6' (*P. domestica* × *P. cerasifera*) and 'Dospina 235' (*P. domestica* × *P. spinosa*) were used for establishing *in vitro* cultures.

Initial culture of Cherry plum was established using leaf buds excised from branches taken at the end of January and maintained in laboratory conditions until breaking dormancy. As regards 'Docera' and 'Dospina', actively growing leaf buds selected from screenhouse-grown plants at the end of March were used as initial explants. Surface sterilization procedure of all explants involved washing explants under tap water for 1.5-2 h, sterilization in 70% ethanol (1 min) and 10% (v/v) commercial bleach solution (10 min), followed by triple rinsing with sterile water. Buds (0.3-0.8 *cm* large) were isolated under the stereomicroscope and placed onto MS medium (Murashige & Skoog, 1962) containing 2 *mg* l^{-1} 6-benzyladenine (BA), 0.5 *mg* l^{-1} indole-3-butyric acid (IBA) and 0.1 *mg* l^{-1} gibberellic acid (GA_3). The following parameters were monitored: percentage of contamination, percentage of necrotic explants and percentage of explants with leaf rosettes initiation. Upon establishment of aseptic culture, uniform shoots of all three genotypes were multiplied on MS medium containing 1 *mg* l^{-1} BA, 0.1 *mg* l^{-1} IBA and 0.1 *mg* l^{-1} GA_3. All media contained 30 *g* l^{-1} sucrose and 7 *g* l^{-1} agar with pH value adjusted to 5.7 before autoclaving at 121 °C, 150 kPa for 20 min. Shoots were repeatedly subcultured for five times at a constant four-week subculture interval. Multiplication parameters (multiplication index and length of axial and lateral shoots) were determined at the end each subculture.

MS medium with mineral salts reduced to ½-strength and organic complex unchanged was used in rooting stage. For all genotypes, rooting treatment included 1 *mg* l^{-1} IBA and 0.1 *mg* l^{-1} GA_3. The percentage of rooted plants, as well as the number and length of roots, and height of the rooted plants were determined after four-week interval.

Shoot cultures were grown in 100 *ml* culture vessels containing 50 *ml* of multiplication medium, at 23 ± 1°C and 16 h-photoperiod (light intensity, 8.83 *W* m^{-2}).

All data were analyzed by ANOVA, followed by the Duncan's Multiple Range Test ($p < 0.05$) for mean separation. Data presented in the form of percentage were subjected to arcsine transformation.

Results and Discussion

The most important step for establishment of aseptic culture is sterilization of initial explants. Sodium hypochlorite applied either alone or in combination with ethanol is usually used disinfectants in plant tissue culture and there are many reports on its successful application for surface sterilization of initial explants (Grant & Hammatt, 1999; Debnath, 2004). In our experiment, 10% commercial bleach, as the source of sodium hypochlorite, was ineffective in disinfecting explants derived from screenhouse-grown plants ('Docera' and 'Dospina'). Contamination rates of explants ranged between 40.0% ('Docera') and 45.9% ('Dospina') (Tab. 1). According to Hartman & Kester (1983) contamination is influenced by growth conditions of mother plants that should be grown in greenhouse under disease- and insect-free conditions, at low humidity and watered so as to avoid overhead irrigation. As for Cherry plum, rate of contamination was significantly lower (4.1%) probably due to the low level of surface contamination of mother plant branches that were kept

under laboratory conditions. In addition, the toxicity to tissues caused by sodium hypochlorite in our experiment was also high in all genotypes and ranged between 20.0% and 35.2% (Tab.1). Therefore, the percentage of explants that initiated leaf rosettes varied in wide range with highest level achieved in Cherry plum (63.3%). The lowest rate of explants initiation was obtained in 'Dospina' (18.9%). However the quality of initiated leaf rosettes was satisfactory and enabled us to establish further experiments (Fig. 1).

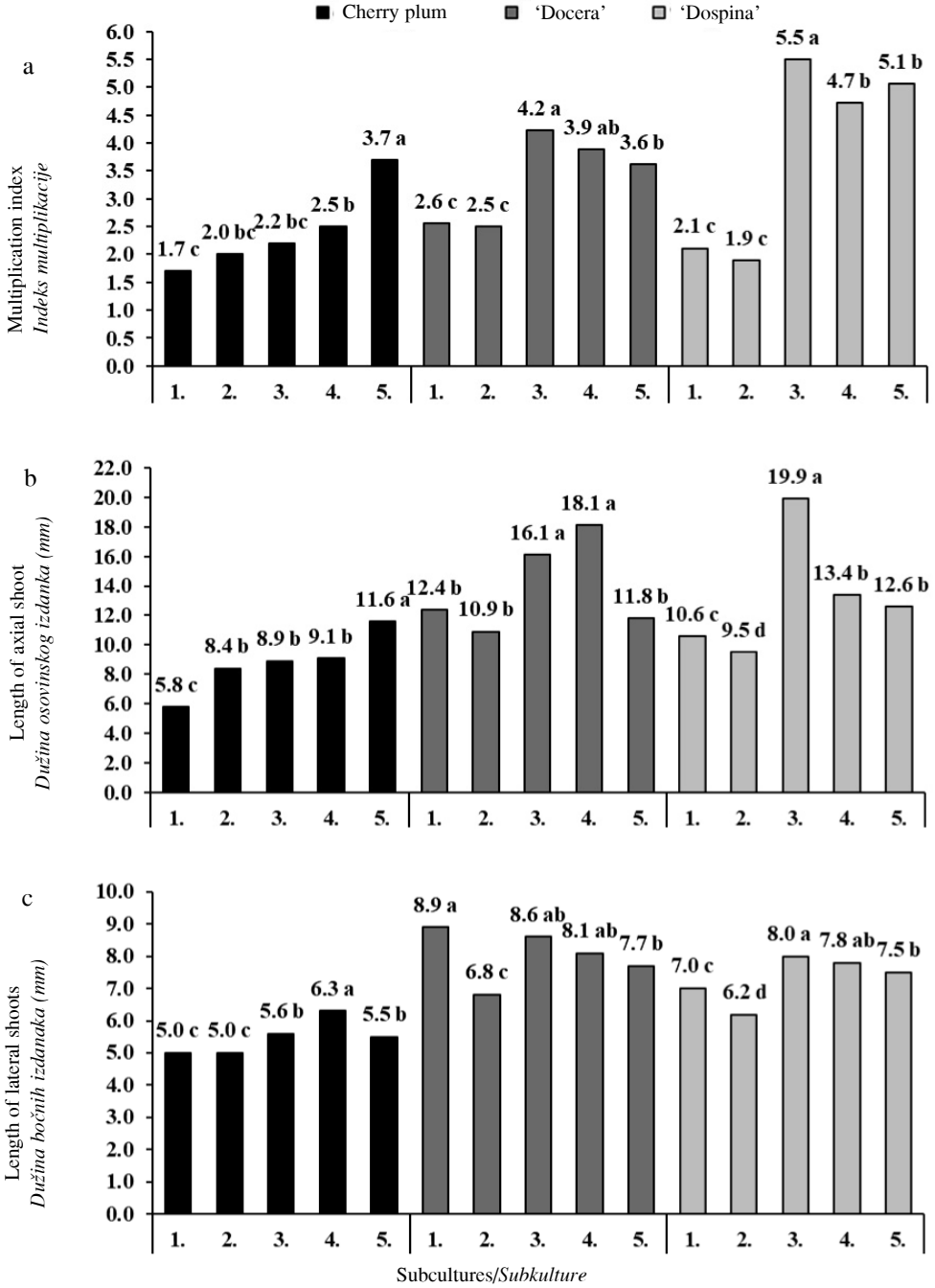
Following establishment of aseptic cultures shoots were multiplied on medium of constant PGR composition over five repeated subcultures. Monitoring of the shoot formation capacity revealed constant increase in multiplication index and length of axial shoots in Cherry plum during each consecutive multiplication cycle (Graph 1). Thus, the highest values of both parameters were achieved in the fifth subculture (multiplication index – 3.7; length of axial shoots – 11.6 *mm*). Hamad & Taha (2008) also reported that the subcultures improved shoot elongation at short-lasting incubation (30 or 45 days). As for 'Docera' and 'Dospina', shoot multiplication index and length of axial shoots significantly increased over first subcultures reaching the highest values in the third subculture ('Docera' – 4.2 and 18.1 *mm*; 'Dospina' – 5.5 and 19.9 *mm*, respectively) and then slightly declined in next two subcultures but still stayed higher compared to first two subcultures (Graph 1). Norton & Norton (1986) also reported the similar decrease in multiplication capacity, shoot length and leaf size after several subcultures in 6 ornamental species and cultivars of *Rosaceae* family. According to the authors, irreversible decline could be either due to genetic or epigenetic change resulting from repeated fluxes in cytokinin, nutrient status or sucrose, or to elimination of seasonal environmental fluctuations occurring *in vivo* (Norton & Norton, 1986). As regards the length of lateral shoots, this parameter also significantly varied in repeated subcultures reaching the highest value in different subcultu-

Tab. 1. Establishment of aseptic culture of plum rootstocks
Tab. 1. Uspostavljanje aseptične kulture podloga za šljivu

Genotype/*Genotip*	Contamination *Procenat kontaminacije* (%)	Necrosis *Procenat nekroze* (%)	Rosette nitiation *Inicijacija rozete* (%)
Cherry plum/*Džanarika*	4.1 b*	32.7 a	63.3 a
'Docera'	40.0 a	20.0 b	40.0 b
'Dospina'	45.9 a	35.2 a	18.9 c

* Mean values for each parameter followed by the same letter are not significantly different according to Duncan's Multiple Range Test ($p < 0.05$)/*Prosečne vrednosti za svaki parametar praćene istim malim slovom nisu značajno različite prema Dankanovom testu višestrukih intervala ($p < 0,05$)*

Fig. 1. Rosette initiation: a) Cherry plum; b) 'Docera'; c) 'Dospina'
Sl. 1. Inicijacija rozete: a) Džanarika; a) Docera; b) Dospina



Mean values for each parameter for each genotype followed by the same letter are not significantly different according to Duncan's Multiple Range Test ($p < 0.05$)/*Prosečne vrednosti za svaki parametar svakog genotipa praćene istim malim slovom nisu značajno različite prema Dankanovom testu višestrukih intervala ($p < 0,05$)*

Graph 1. Effect of subculturing on multiplication parameters of plum rootstocks
Graf. 1. Uticaj supkultivisanja na parametre multiplikacije podloga za šljivu

re for each genotype (the fourth subculture in Cherry plum, the first in 'Docera' and the third in 'Dospina') (Graph 1). No visible morphological variations or aberrations of shoots were found in successive subcultures in any genotype. Shoots were well developed with wide, dark green leaves (Fig. 2).

Rooting capacity of *in vitro* shoots depends on different factors: explants age, total time spent in culture, type and concentration of cytokinins i.e. cytokinin/auxin ratio applied in multiplication stage, as well as on amount of cytokinins in tissues adopted during the multiplication phase. Taking into account all analyzed rooting parameters, the highest rooting ability among genotypes was observed in Cherry plum and the lowest in 'Dospina' (Graph 2), although rooted shoots of each rootstock were vigor with well developed and strong root system (Fig. 3). Therefore, an additional optimization of this stage by testing other types and concentration of auxin should be done in order to achieve higher rooting percentage in 'Dospina'.

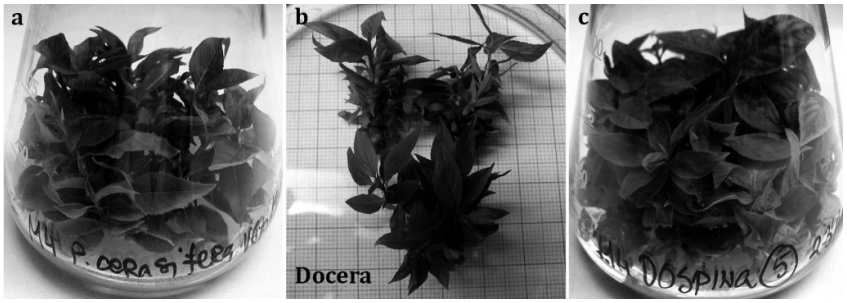
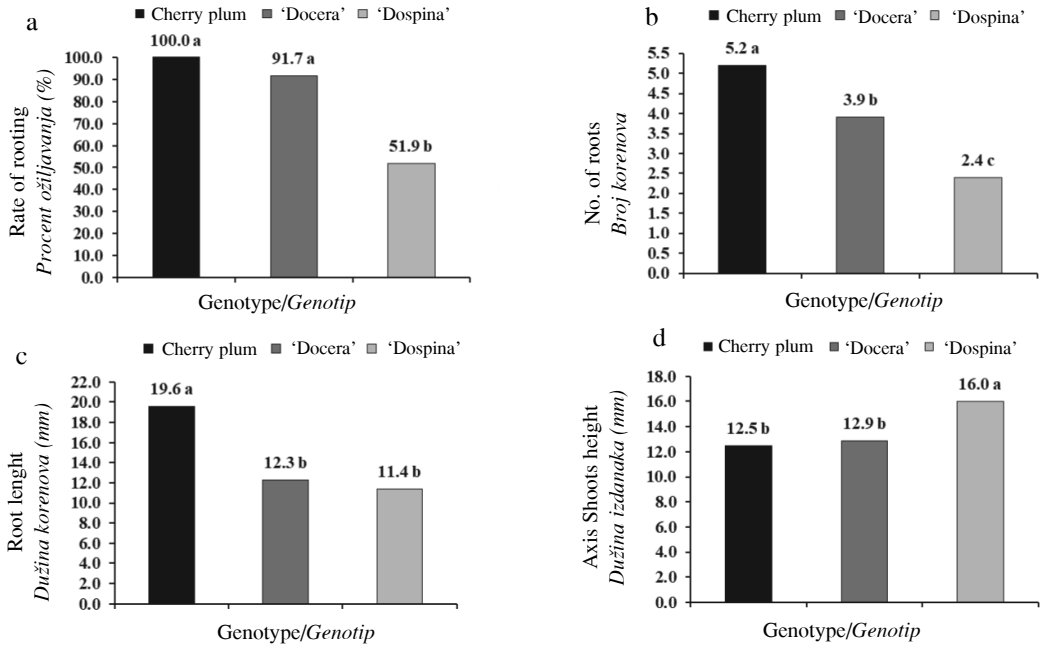


Fig 2. Shoots of plum rootstocks in multiplication stage: a) Cherry plum; b) 'Docera'; c) 'Dospina'
Sl. 2. Izdanci podloga za šljivu u fazi multiplikacije: a) Džanarika; b) Docera; c) Dospina



Mean values for each parameter followed by the same letter are not significantly different according to Duncan's Multiple Range Test ($p < 0.05$)/*Prosečne vrednosti za svaki parametar praćene istim malim slovom nisu značajno različite prema Dankanovom testu višestrukih intervala ($p < 0,05$)*

Graph 2. Parameters of rooting in plum rootstocks
Graf. 2. Parametri ožiljavanja pologa za šljivu

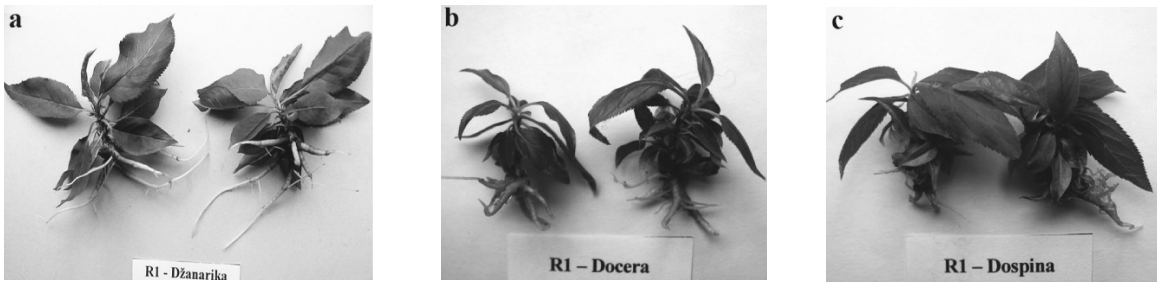


Fig 3. Shoots of plum rootstocks in rooting stage: a) Cherry plum; b) 'Docera'; c) 'Dospina'
Sl. 3. Izdanci podloga za šljivu u fazi ožiljavanja: a) Džanarika; b) Docera; c) Dospina

Conclusion

This study has confirmed that response of *in vitro* cultures to constant subculturing is genotype dependant. Cherry plum has shown increase in regeneration ability during five successive subcultures, while in 'Docera' and 'Dospina' shoot multiplication index and shoot length increased during subculturing up to the third subculture, and then slightly decreased. The decline in shoot multiplication rate implies necessity for further investigation in order to find the proper method of restoring regeneration capacity of *in vitro* shoots and/or delay the decline for several subcultures. Considering that the decline in multiplication index was already observed after third subculture, it is neccesary to determine if and when cytokinin type and concentration should be reduced, or if hormon-free medium should be deployed to delay the decrease.

Acknowledgements

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IN VITRO RAZMNOŽAVANJE PODLOGA ZA ŠLJIVU

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Rezime

Tri podloge za šljivu, džanarika (*Prunus cerasifera* Ehrh.), Docera 6 (*P. domestica* × *P. cerasifera*) i Dospina 235 (*P. domestica* × *P. spinosa*) su umnožavane *in vitro* tokom pet uzastopnih supkultura na Murashige & Skoog (MS) hranljivoj podlozi konstantnog sadržaja biljnih regulatora rastenja u cilju ispitivanja uticaja sukcesivnog supkultivisanja na parametre multiplikacije.

Praćenjem regenerativnog kapaciteta utvrđeno je konstantno povećanje indeksa multiplikacije, dužine osovinskih i bočnih izdanaka kod podloge džanarika. Kod podloga Docera i Dospina uočeno je značajno povećanje indeksa multiplikacije i dužine osovinskih izdanaka u trećoj supkulturi, posle čega njihova vrednost postepeno opada do pete supkulture, ali i dalje ostaje značajno veća u odnosu na prve dve supkulture. Ovi rezultati potvrđuju činjenicu da uticaj supkultivisanja na kapacitet za multiplikaciju *in vitro* značajno varira u zavisnosti od genotipa. Takođe, uzimajući u obzir da je kod podloga Docera i Dospina pad regenerativnog kapaciteta izražen, kroz indeks multiplikacije, uočen već posle treće supkulture, neophodno je precizno definisati da li se i kada vrsta i koncentracija primenjenih citokinina mora promeniti ili pak upotrebiti hranljiva podloga bez hormona u cilju odlaganja ili prevazilaženja ovog smanjenja.

U fazi ožiljavanja je korišćena MS hranljiva podloga sa mineralnim solima smanjenim na ½ i organskim kompleksom nepromenjenim prema MS. Najveći kapacitet ožiljavanja *in vitro* utvrđen je kod podloge džanarika (100%), pa zatim kod podloge Docera (91,7%), a najniži kod podloge Dospina (51.9%). Stoga je kod podloge Dospina potrebno izvršiti dodatnu optimizaciju ove faze testiranjem drugih vrsta auksina u cilju postizanja većih procenata ožiljavanja.

Ključne reči: džanarika, Docera, Dospina, *in vitro*, mikropropagacija