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Rapid Propagation of Sweet and Sour Cherry Rootstocks

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Abstract

The paper presents a protocol for micropropagation of *Prunus* sp. rootstocks included in the sweet and sour cherry breeding program. Germplasm diversity for rootstock breeding derives from natural populations, where conditions and biological vectors for systematic infection with viral diseases are constantly present. The establishment of aseptic culture depends primarily on the explant type, as all selections were collected from natural habitat. For nearly all investigated selections, dormant buds were the favored source, due to enabling rosette initiation in more than 58% cases. In *P. cerasus* L. selections, 100% contamination was noted when shoot tips were used as an explant source. Significant influence of the double-phase medium on the number and height of multiplied shoots was observed in the standard cherry rootstock, 'Gisela 6'. For *P. fruticosa* Pall., selection 'SV1' and 'SV2', and *P. cerasus* 'D6' selection, the double-phase medium also had a significant effect on the height of multiplied shoots, when compared to solid DKW (Driver and Kuniyuki Walnut) medium. Genetic variability of selections within the investigated species resulted in variable plant rooting success. Adding Fe-EDDHA (Ethylenediamine di-2-hydroxy-phenyl acetate ferric) in the 200 mg l^{-1} concentration to the rooting medium significantly enhanced the percentage of rooted plants. The highest rooting percentage was noted for 'Gisela 6' and 'D6' genotype at 1 mg l^{-1} IBA (indole-3-butyric acid), while 0.8 mg l^{-1} was the optimum concentration for *P. mahaleb* L. 'M1' selection. *P. fruticosa* genotypes required significantly higher IBA concentration for rooting (2.5 and 3.5 mg l^{-1}).

Keywords: clonal multiplication, juvenility, plant growth regulators, tissue culture

Abbreviations: BA: 6-benzyladenine; DKW: Driver and Kuniyuki Walnut medium; Fe-EDDHA: Ethylenediamine di-2-hydroxy-phenyl acetate ferric; GA_3: Gibberellic acid; IBA: Indole-3-butyric acid; MS: Murashige and Skoog; NAA: 1-naphtaleneacetic acid; SH: Schenk and Hildebrandt

Introduction

Every potential new rootstock has to be subjected to a long-term evaluation process that requires considerable space, time and knowledge, as well as extensive financial investment (Ljubojević *et al*., 2013). Selection success in breeding of vegetative rootstocks for sweet and sour cherry is dependent not only on genetic variability, but also on expediting propagation processes as a precondition for investigation of specific combining abilities between rootstock and scion (Bošnjaković *et al*., 2012b; Bošnjaković *et al*., 2013).

Biodiversity of wild fruit species provides an inexhaustible gene pool for breeding work in *Prunus* sp. (Paunović, 2008; Bošnjaković *et al*., 2012a). There is a tremendous opportunity for genetic improvement and utilization of *P. mahaleb* L., *P. cerasus* L., and *P. fruticosa* Pall. genotypes to facilitate rootstock breeding. Conventional germplasm preservation and clonal propagation activities in rootstock breeding mandate that several years are dedicated to *ex situ* conservation and clonal propagation by stool beds, soft or hardwood cuttings. *In vitro* plants can be used for *ex situ* conservation of genetic resources on their own roots, thus replacing standard propagation by grafting.

Micropropagation is a widely used method for multiplication of standard rootstocks, as well as for new plant cultivars and rootstock selections (Dradi *et al*., 1996; Pruski *et al*., 2005; Vujović *et al*., 2012; Druart, 2013). However, it is possible to propagate plants throughout the entire year, whereby the multiplication rate significantly

exceeds that of vegetative propagation by cuttings. As a universal medium for *in vitro* plants does not exist, selection of appropriate medium, plant growth regulators and other components that could enhance plant production is very important (Ružić *et al*., 2000; Rustaei *et al*., 2009; Hossini *et al*., 2010). Some authors recommend adding activated charcoal into the liquid medium as a means to enhance elongation and multiplication rate (Jona and Vigliocco, 1985; Hassan and Roy, 2005), while some others posit that supplementation by different iron sources can increase the number of rooted plants (Dimassi *et al*., 2003; Molassiotis *et al*., 2003; Molassiotis *et al*., 2004; Trejgell *et al*., 2012). Presently, there is paucity of studies investigating the effect of introducing iron into the rooting medium for sweet and sour cherry rootstocks. Aghaye *et al*. (2013) reported achieving 100% rooting in 'Gisela 6' using different thiamine and Fe-EDDHA concentrations. As sweet cherry rootstocks are cited as difficult to root (Feucht and Dausend, 1976; Štefančić et *al*., 2007; Sedlak *et al*., 2008), an efficient propagation method of new rootstock selections is needed.

The aim of this study was to develop protocol for micropropagation of selections included in the rootstock breeding program discussed in this work. The focus was on examining the influence of the double-phase medium on the multiplication index as well as the effects of Fe-EDDHA on rooting of rootstock selections for sweet and sour cherry.

Materials and methods

Plant material and culture establishment

The dormant buds and actively growing shoot tips of 'Gisela 6' and five promising rootstock selections were used as initial explants. Dormant buds were collected at the end of December, while shoot tips were obtained in May from mature trees grown *ex situ* in the experimental field at the Faculty of Agriculture, Novi Sad.

The rapid multiplication and rooting ability of 'Gisela 6' and five selections – labeled as 'SV1' and 'SV2' from *P. fruticosa* (hereinafter referred to as 'SV1' and 'SV2'), 'M1', from *P. mahaleb* (henceforth - 'M1'), and 'D3' and 'D6' from *P. cerasus* (henceforth - 'D3' and 'D6') – were investigated. The surface sterilization was carried out using two different protocols – for dormant buds, and for actively growing shoot tips. Dormant twigs with one node were first rinsed in tap water for 2 hours, after which they were disinfected first – with fungicide Previcur (5%) for 30 minutes, before being immersed in 0.1% mercuric chloride for 5 minutes. Sterilization was accomplished in laminar flow hood with 70% ethanol containing 0.1% Tween for 1 minute, followed by being placed in 4% sodium hypochlorite for 25 minutes, and finally subjected to 3 rinses with sterile distilled water. After peeling off the outer scales, the buds were again immersed in 70% ethanol, and 4% sodium hypochlorite, this time for 5 minutes, followed by two rinses with sterile distilled water. Meristem dissection with 2-3 primordial leaves was performed under stereomicroscope and cultured on the initiation medium containing SH (Schenk and Hildebrandt, 1972) macroelements, MS (Murashige and Skoog, 1962) microelements and vitamins, with (in mg l^{-1}) 6-benzyladenine (BA) 0.5, 1-naphtaleneacetic acid (NAA) 0.01 and gibberellic acid (GA_3) 0.5 mg l^{-1}. Sucrose (30 g l^{-1}) was used as a carbon source, solidified with 6.5 g l^{-1} agar, with the pH value adjusted to 5.8 before autoclaving at 121 °C and 100 kPa for 25 minutes.

Shoot tip sterilization followed the standard procedure, whereby the explants were excised to 1.5 cm height, before being washed under running tap water for 2 hours, and disinfected first – with Previcur (5%) for 30 minutes, and later using mercuric chloride (0.1%) for 5 minutes. Under a sterile laminar flow hood, actively growing shoot tips were sterilized with 70% ethanol, containing 0.1% Tween for 1 minute, and in 4% sodium hypochlorite for 25 minutes. After rinsing in sterile distilled water three times, explants were cultured on the same initiation medium as dormant buds.

After three weeks on the initiation medium, the explants infection, initiation and browning rates were measured.

Multiplication

Multiplication parameters of the newly formed shoots were investigated on the solid and double-phase DKW (Driver and Kuniyuki, 1984) medium. Solid medium was supplemented with BA and indole-3-butyric acid (IBA) in the concentration of 0.8 mg l^{-1} and 0.01 mg l^{-1}, respectively, and sucrose and agar in the concentration of 30 g l^{-1} and 6.5 g l^{-1}, respectively. Liquid medium contained double concentration of DKW macroelements, microelements and vitamins, as well as half-strength BA concentration, supplemented with 2 g l^{-1} active charcoal, with the pH value adjusted to 5.8 before autoclaving at 121 °C and 100 kPa for 20 minutes. Liquid medium did not comprise agar and sucrose. Approximately 20 ml of liquid medium was added on the top of the solid medium. Multiplication index, length of the multiplied shoots exceeding 0.5 cm, number of leaves, leaf width and leaf length were measured after four weeks on solid and double-phase multiplication medium.

Rooting

After multiplication on the solid medium, plants were placed on DKW hormone-free medium, aimed at improving shoot elongation before rooting. Shoots of the investigated genotypes were rooted on half-strength macro and micro MS elements, and vitamins, supplemented with 20 g l^{-1}sucrose and 6.5 g l^{-1} agar. Depending of genotype, different IBA (0.5-3.5 mg l^{-1}) concentrations were used, and Fe-EDDHA in the concentration of 200 mg l^{-1} was added as an iron source. The pH of the medium was adjusted to 5.8 before autoclaving. After four weeks, the rooting percentage, number of roots and total root length were recorded. All cultures were incubated in an environmental chamber at 24±2 °C with 16 h photoperiod.

Statistical analysis

All collected data were analyzed by ANOVA, followed by the Duncan's multiple range tests, performed by STATISTICA 10.0 (StatSoft, Inc., Tulsa, OK, USA). Number of roots and root length were measured by 'ImageJ 1.44p' (Wayne Rasband, National Institute of Health, USA).

Results and discussions

Making a correct choice of explants type has an important effect on the success of further tissue culture. Since all collected plant material used in this study originated from *ex situ* conditions, contamination was observed at all genotypes. Initiation from actively growing shoot tips, for 'D3' and 'D6', seems to be the least favourable choice, as it resulted in infection in 100% of the explants (Tab. 1). However, the advantages of using shoot tips over dormant buds include greater survival rate to *in vitro* conditions, more rapid growth onset, and a greater number of produced shoots. On the other hand, explants of a greater size tend to be more difficult to decontaminate (George and Debergh, 2008). With the exception of 'SV1', high percentage of contaminated explants was found in all other selections (79.3-95.0%). Even though *in vitro* cultures can be established at any time of the year, the success rate depends on the season of explant collection (Dobránszki and Teixeira da Silva, 2010; Ružić *et al*., 2010). In buds 0.2-0.6 cm in length, Kaushal *et al*. (2005) reported 60% survival rate, while nearly all 1-2 cm long explants died during subculturing. These findings are in line with our results pertaining to dormant buds, where rosette initiation success rate exceeded 57.7% in all cases, with the exception of 'D6' genotype. For apricot, Perez-Tornero *et al*. (1999) recommended establishing explants from meristem tip culture, indicating that, this technique has a potential for decreasing prevalence of explant infection. Our results indicate that winter months are more suitable for establishing explants *in vitro*, enabling higher rosette initiation, and achieving better multiplication scheduling. Until the end of December, due to the greater cumulative number of chilling hours, the growth inhibitor abscisic acid levels declined. However, the amount of both cytokinins and giberrelins subsequently increased, reaching the levels sufficient for normal shoot growth (Pruski *et al*., 2005). Meristem tip was the most suitable explant for cultures initiated from field-grown mother trees, which are putatively contaminated (Druart, 2013). Moreover, this timing enables transferring acclimatized plants into the field during spring. Explant browning was present when dormant buds were used as an explant source, possibly due to repeated disinfection with ethanol and sodium hypochlorite, which resulted in plant tissue damage. The most pronounced browning was observed in 'SV1' genotype (27.0%).

Significantly better influence of double-phase medium on the number and height of newly formed shoots was noted at standard cherry rootstock 'Gisela 6' (Tab. 2). No significant differences among the multiplication index and height of the newly formed shoots between solid and double-phase medium were noted in 'D3' and 'M1' genotype. In contrast, shoot height of the 'D6' genotype was considerably affected by the addition of liquid medium. Plant height is very important parameter during acclimatization and survival of multiplied plants. After the multiplication phase, in the previously described treatment, Bošnjaković *et al*. (2013) rootstock selections were placed on DKW hormone-free medium, aimed at improving shoot elongation before rooting. Although some authors reported achieving shoot elongation prior to rooting in the treatments based on BA lower levels (Zilkah *et* al., 1992; Szczygiel and Wojda, 2010), any improvements in shoot elongation could not observe (unpublished data). However, DKW hormone-free medium had a positive influence on the elongation of multiplied shoots, in line with Pruski *et al.* (2005). The present results show that using the double-phase medium, it is possible to reduce the time and *in vitro* manipulation during subculturing, thus eliminating the phase of subculturing to fresh medium. This finding is in line with the results reported by Dobránszki and Teixeira da Silva (2010) and Scherwinski-Pereira *et al*. (2012). In addition, these authors pointed out that usage of liquid medium could reduce the micropropagation cost, which is an important parameter in commercial plant propagation. Moreover, in double-phase medium, the explants take up nutrients, hormones, and plant growth regulators faster and from both layers (Gupta *et al*., 2005; George and Davids, 2008; Scherwinski-Pereira *et al*., 2012).

In 'SV1' and 'SV2', while a greater multiplication index (5.4 and 6.7, respectively) was achieved on solid medium, using the double-phase medium resulted in a significant increase in newly formed shoot height (by 2.5 and 2.8, respectively). Positive effects of using a double-phase medium on multiplication and height of multiplied shoots was investigated in pineapple (Scherwinski-Pereira *et al*., 2012), vanilla (De Oliveira *et al*., 2013), apple (Litwinczuk, 2000), and cherry rootstocks (Dziedzic and Malodobry, 2006). In this study, only 'M1' was negatively affected by the use of double-phase medium as the newly formed shoots started to yellow and vitrify. Paquez and Boxus (1987) reported higher vitrification and production of dry weight in larger quantities in samples grown on liquid than those maintained on a similar solid medium. *P. mahaleb* is well known for its susceptibility to water logging in *in vivo* conditions as well. The growth and development of shoots was affected by addition of active charcoal to both liquid and solid medium, due to its capacity for adsorbing the inhibitory substances in culture medium, as well as the excess cytokinins (Jona and Vigliocco, 1985; Pan and van Staden, 1998; Thomas, 2008). Positive effects of active charcoal in micropropagation of *Prunus* rootstocks were also reported by Dradi *et al*. (1996), Szczygiel and Wojda (2010) and Druart (2013). Number of leaves reflects the shoot quality and thus its potential for further subcultivation. In 'D3' and 'D6' genotypes, no significant differences in the numbers of leaves, leaf length and leaf width were noted between solid and double-phase medium. On the other hand, significantly higher number of leaves was found in 'Gisela 6' when double-phase medium was used. In 'SV1' and 'SV2', as well as 'M1', the number of leaves was higher on solid than double-phase

Tab. 1. Establishment of aseptic culture from shoot tips and dormant buds

Genotype	Type of explants	Rosette initiation (%)	Infected explants (%)	Explant browning (%)
'Gisela 6'	Shoot tip	12.3bc	87.7ab	-
	Dormant bud	75.0a	10.0d	15.0bc
'D3'	Shoot tip	0.0c	100.0a	-
	Dormant bud	57.7b	31.0b	11.3c
'D6'	Shoot tip	0.0c	100.0a	-
	Dormant bud	7.0c	91.0a	2.0d
'M1'	Shoot tip	5.0c	95.0a	-
	Dormant bud	63.3b	17.0c	19.7b
'SV1'	Shoot tip	48.3a	51.7c	-
	Dormant bud	59.0b	14.0cd	27.0a
'SV2'	Shoot tip	20.7b	79.3b	-
	Dormant bud	71.0a	29.0b	0.0d

Note: Numbers followed by a different letter are significantly different at $P<0.05$, according to Duncan's multiple range tests

medium, although this parameter is genotype-dependent.

Rooting ability differed among investigated species, and among investigated genotypes within the same species. During subculturing, all explants of 'D3' genotype died due to bacterial contamination; thus, rooting trials could not be conducted due to the insufficient number of plants. For all investigated genotypes, Fe-EDDHA was added to the rooting medium in the concentration of 200 mg l^{-1}, as this enables better rooting, produces plantlets of higher quality, and promotes correction of shoot-tip necrosis. Iron is required for chlorophyll biosynthesis, and its deficiency is often manifested as leaf chlorosis (Dunlap and Robacker, 1988; Gaspar *et al*., 1992), which can lead to necrosis (van der Salm *et al*., 1994). Ca content is one of the most important factors for the development of shoot tip necrosis (Bairu *et al*., 2009). It has been established that the application of iron results in an increase in the Ca content in the peach leaf (Fernández *et al*., 2008), which can explain the positive effect of iron on the reduction in the prevalence of shoot tip necrosis. The auxin produced in the shoot, basipetally transported towards the root system (Ljung *et al*., 2001), plays a crucial role in the root development (Aloni *et al*., 2006). Shoot tip necrosis most likely disrupts the auxin production and flow, which negatively affects the rooting. This effect was previously reported by Xing *et al*. (1997), whose study demonstrated that higher rooting rate was associated with lower prevalence of shoot-tip necrosis.

At the same IBA concentration, adding Fe-EDDHA to the rooting medium resulted in a significantly higher rooting percentage in 'Gisela 6' and 'D6' genotype (Tab. 3). As 'Gisela' rootstock series is mainly propagated by micropropagation, a successful protocol for 'Gisela 6' was reported by Vujović *et al*. (2009), Hossini *et al*. (2010), Aghaye *et al*. (2013) and Sarropoulou *et al*. (2013), who achieved rooting percentage exceeding 80%, with 1 mg l^{-1} IBA concentration, which is in the line with the present results.

In our study, 'M1' selection has shown maximum rooting ability, and has produced both the highest number of roots and the greatest total root length at 0.8 mg l^{-1} IBA concentration, which is in line with the findings of Dradi *et al*. (1996). On the other hand, in *P. fruticosa* genotypes, satisfactory rooting was achieved at significantly higher concentrations of auxins – for 'SV1' and 'SV2', the optimum concentration was 3.5 mg l^{-1} and 2.5 mg l^{-1} IBA, respectively (Tab. 3). When lower concentrations of IBA were used, genotypes tended to show high cell division, producing callus. However, roots were either absent, or present in a very small number, with evident symptoms of shoot-tip necrosis. In 'SV1' genotype, Fe-EDDHA significantly increased the percentage of rooted plants (from 60% to 85.6%), when 2 mg l^{-1} IBA concentration was used, while at the 1 and 2 mg l^{-1} IBA, without Fe-EDDHA, rooting was not achieved in 'SV2'. While increasing the IBA concentration increased the percentage of rooted plants, it was significantly higher in the presence of Fe-EDDHA. Pruski *et al*. (2005) reported that using the combination of IBA and NAA resulted in 79% rooting for *P. fruticosa*, while Szczygiel and Wojda (2010) achieved only 34% of rooted plants with 2 mg l^{-1} IBA. Increasing auxin concentration resulted in a somewhat increased number of roots and total length. In 'M1' and 'SV2', maximum number of roots, as well as the greatest total root length, was noted at IBA concentrations of 0.8 and 3 mg l^{-1},

Tab. 2. Multiplication parameters on solid (S) and double-phase (DP) medium

Genotype	Multiplication index		Height of proliferated shoots (cm)		Number of leaves		Leaf length (cm)		Leaf width cm)	
	S	DP	S	DP	S	DP	S	DP	S	DP
'Gisela 6'	2.7de	3.9c	2.2c	3.5a	15.2def	19.3b	1.5a	1.2d	0.8ab	0.8b
'D3'	1.8f	1.7f	1.0e	0.8ef	17.5bcd	17.9bc	1.3bc	1.4b	0.5e	0.6de
'D6'	1.9ef	2.4def	1.4d	2.0c	15.9cde	16.3cde	1.3bc	1.4b	0.5e	0.5e
'M1'	3.0d	2.2def	0.9ef	0.7f	17.5bcd	13.4f	1.0e	1.2cd	0.9a	0.6d
'SV1'	5.4b	3.9c	0.9ef	2.5b	23.7a	17.4bcd	1.3bcd	1.3bc	0.5e	0.5e
'SV2'	6.7a	4.1c	1.4d	2.8b	18.2bc	14.6ef	1.3bcd	1.4b	0.5e	0.7c

Note: Numbers followed by a different letter are significantly different at P<0.05, according to Duncan's multiple range tests

Tab. 3. Effect of auxins and Fe-EDDHA on rooting percentage, number of roots and total root length

Genotype	IBA (mg l^{-1})	Fe-EDDHA (mg l^{-1})	Percent of rooting (%)	Number of roots	Total root length (cm)
'Gisela 6'	1	/	61.7e	6.9efghi	7.5efg
	1	200	92.7ab	10.9bcd	13.9cd
'D6'	1	/	42.0f	8.0defgh	9.3defg
	1	200	60.0e	8.9cdefg	11.0def
'M1'	0.5	200	83.9bcd	5.2hi	7.0efg
	0.8	200	96.7a	8.9cdefg	8.8defg
	1	200	80.0d	6.5efghi	4.4g
'SV1'	0.5	/	6.9i	4.1i	12.3cdef
	1	/	29.4g	3.6i	10.8def
	2	/	60.0e	4.5i	7.0efg
	1	200	18.3h	6.0fghi	8.8defg
	2	200	85.6bcd	9.1cdef	12.0cdef
	2.5	200	82.7bcd	9.1cdef	10.6def
	3	200	85.0bcd	9.6cde	12.7cde
	3.5	200	91.8abc	13.7b	16.9bc
'SV2'	0.5	/	0	/	/
	1	/	0	/	/
	2	/	0	/	/
	1	200	5.6i	6.5efghi	6.1fg
	2	200	18.3h	5.9fghi	8.5defg
	2.5	200	85.0bcd	5.6ghi	10.5defg
	3	200	80.0d	18.9a	33.1a
	3.5	200	81.7cd	11.6bc	20.7b

Note: Numbers followed by a different letter are significantly different at P<0.05, according to Duncan's multiple range tests

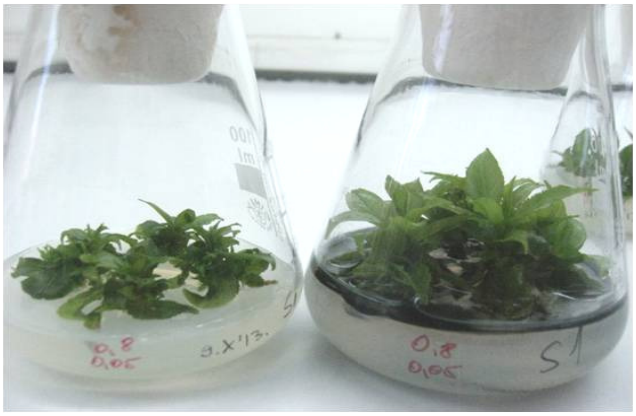

Fig. 1. The differences in the effect of the solid and double-phase multiplication medium applied to *P. fruticosa* 'SV1' selection on the proliferated shoot height

respectively, after which a decline was observed. In contrast, in 'SV1', maximum number of roots and total root length was measured at the highest applied IBA concentration (Tab. 3). Similar results were reported by Shibli *et al*. (1997), who stated that number of roots and root length increased as the IBA concentration increased. However, according to other authors, high concentrations of applied auxins could inhibit root growth and elongation (Loach, 1988; Taiz and Zeiger, 2006; Zhao *et al*., 2014). In 'Gisela 6' and 'D6', greater number of roots and total root length was measured in the presence of Fe-EDDHA, thus conforming to the results of Dimassi *et al*. (2003). Iron is a part of peroxidase, an enzyme responsible for adventitious root formation (Gaspar *et al*., 1992; Hatzilazarou *et al*., 2006). Positive effects of Fe-EDDHA on rooting of peach rootstock GF-677 were reported by Molassiotis *et al*. (2003), Balla and Kirilla (2006), Antonopoulou *et al*. (2007) and Hasan *et al*. (2010).

Fig. 2. The effect of Fe-EDDHA on the rooting of *P. fruticosa* 'SV2' selection: a) without Fe-EDDHA plants showing symptoms of shoot tip necrosis, b) with Fe-EDDHA plants with well developed roots

Conclusion

As micropropagation is a process characterized by an interaction of different components, it is difficult to optimize the culture medium for investigated germplasm. Thus, it is not possible to establish a uniform protocol that can be applied to propagation within and between species, as it is strongly genotype-dependent. Thus, the effect of the medium should be studied for each genotype separately. Considering the fact that standard rootstocks for sweet and sour cherry are mostly propagated by micropropagation, vegetatively propagated selections of *P. mahaleb*, *P. cerasus* and *P. fruticosa* have been successfully achieved through a large number of attempts to establish cultures *in vitro*. This was necessary, as all samples originated from their natural habitat, and thus subject to uncontrolled contamination. As, in this work, a large number of rooted and acclimatized selections has been achieved, the micropropagation protocol has been optimized. The protocols discussed here were given a priority over softwood cuttings, as the aim was to establish a faster process for implementing newly propagated selections in a breeding program. The vegetative propagation method investigated in this study presents the best and the fastest genotype propagation method. Ljubojević *et al*. (2013) proved that dwarf rootstocks are a promising material, due to their anatomical, morphological and physiological parameters. This propagation method provided sufficient number of rootstocks with special combining abilities, which are currently being tested in field grafting trials.

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