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Use of in vitro propagation of 'Oblačinska' sour cherry in rootstock breeding

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Abstract: *Prunus cerasus* L. 'Oblačinska' sour cherry germplasm was established in vitro directly from in situ plants on different explant collection dates, enabling quick clonal multiplication and introduction to a rootstock breeding program. Rosette initiation of four investigated genotypes was possible from November to April on the medium containing Schenk and Hildebrandt (SH) macroelements, Murashige and Skoog (MS) microelements, and vitamins supplemented with (in mg L^{-1}) 6-benzyladenine (BA), 0.5; indole-3-butyric acid (IBA), 0.01; gibberellic acid (GA_3), 0.1; citric acid, 10; and L-ascorbic acid, 10. The lowest percent of contamination was noted in November and December, when dormant buds were used as an explant source, and the highest percentage was found with actively growing shoot tips. The elongation phase of rosettes initiated from dormant buds remained a major obstacle. Survival rates of shoot tips obtained in April were high and subsequent growth more prominent. An increasing index of multiplication from 1.5 to 1.9 was noted on Driver and Kuniyuki walnut medium (DKW) with 0.8 mg L^{-1} BA and 0.01 mg L^{-1} IBA in the 'OV 32' genotype. Rooting percentages of 71.3% and 81.3% were achieved in 'OV 17' and 'OV 32' genotypes, respectively, on half-strength MS medium with 1 mg L^{-1} IBA.

Key words: Dormant period, elongation, explant collection date, *Prunus cerasus* L., rooting

1. Introduction

The variability in sour cherry germplasm presents a wealthy source of diversity for breeders (Radičević et al., 2012). The Balkan Peninsula is one of the most valuable secondary centers of genetic diversity and provides an inexhaustible gene pool for breeding work in *Prunus* sp. (Ognjanov et al., 2009; Bošnjaković et al., 2012). 'Oblačinska' sour cherry (*Prunus cerasus* L.) has been used for decades as a dwarfing rootstock for sweet and sour cherry in Serbia as a population cultivar, but it has never been of national interest to conserve and utilize germplasm for rootstock breeding (Ognjanov et al., 2012). Population heterogeneity causes major problems in propagation, and selecting and isolating clones with the most favorable characteristics requires work (Nikolić et al., 2005; Miletić et al., 2008). An efficient vegetative propagation method for *P. cerasus* L., including 'Oblačinska' sour cherry, has not been reported, since the main propagation method is from root suckers and seeds. Protocols for micropropagation are presented in Gisela 5 (Vujović et al., 2012), Gisela 6 (Vujović et al., 2009; Hossini et al., 2010), PHL-A (Sedlak et al., 2008; Mahdavian et al., 2011), sour cherry varieties (Cerović and Ružić, 1987), and sweet and sour cherry rootstock selections (Dradi et al., 1996; Singh et al., 2010). The merit of using in vitro meristem, shoot tip, or bud cultures in rootstock breeding, as a means of multiplication, is that the incipient shoot has already been differentiated in vivo. At the same time, as a means of germplasm preservation, it assures the availability of genetic material as the need arises. For plant species with a definite dormant period, the success of culture establishment is affected by the season during which the explants are obtained (Preece, 2008).

Germplasm diversity for rootstock breeding derives from natural populations where infections and viral diseases are constantly present (Dorić et al., 2014). A very important advantage of introducing in vitro germplasm into rootstock breeding is the potential for the recovery of virus-free plants from infected germplasm through shoot meristem culture (Druart, 2013).

The aim of this study was to develop a faster, reliable way to introduce genetic diversity within *Prunus cerasus* L. 'Oblačinska' sour cherry into a rootstock breeding program. The objective was to examine seasonal influence on in vitro culture establishment, index of multiplication, and rooting percentage.

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2. Materials and methods

2.1. Plant material and date of explant collection

The establishment of aseptic culture in vitro for four promising *Prunus cerasus* L. 'Oblačinska' sour cherry accessions—'OV 14', 'OV 15', 'OV 17', and 'OV 32'—was examined. Aseptic cultures were established from plants grown in situ, whereby dormant buds were collected monthly from November to March, and actively growing shoot tips were collected in April.

2.2. Surface sterilization

Dormant buds and shoot tips were first disinfected by rinsing in tap water for 2 h. Dormant twigs with one node were surface-disinfected with the fungicide Previcur (5%) for 30 min and immersed in 0.1% mercuric chloride for 5 min. Sterilization was accomplished in a laminar flow hood with 70% ethanol containing 0.1% Tween for 1 min and 25 min in 4% sodium hypochlorite followed by 3 rinses with sterile distilled water. Meristem dissection with 2–3 primordial leaves was performed under a stereomicroscope. The same surface sterilization procedure was applied to actively growing shoots with excised explants 1.5 cm in length.

2.3. Culture medium

In vitro cultures were initiated on medium containing SH macroelements (Schenk and Hildebrandt, 1972), MS microelements, and vitamins (Murashige and Skoog, 1962) supplemented with (in mg L^{-1}) 6-benzyladenine (BA), 0.5; indole-3-butyric acid (IBA), 0.01; gibberellic acid (GA_3), 0.1; citric acid, 10; and L-ascorbic acid, 10. After two subcultures were grown on the initiation medium, explants were taken on DKW elongation medium (Driver and Kuniyuki, 1984) containing 0.2 mg L^{-1} BA and 0.05 mg L^{-1} IBA. After three subcultures on the elongation medium, explant lengths were measured. Well-developed explants were placed on DKW multiplication medium containing (in mg L^{-1}) BA, 0.8 and IBA, 0.01. After the multiplication phase, single node explants were rooted on half-strength macro and micro MS elements, leaving vitamin content unchanged, while varying the IBA concentrations (0.5–1.5 mg L^{-1}). All medium contained 3% sucrose and 0.65% agar with the pH value adjusted to 6.25 before autoclaving at 121 °C (150 kPa) for 25 min.

After 20 days in culture, infection, initiation, and explant development were measured. After the first and second subculture on the multiplication medium, number and length of proliferated shoots were recorded. Rooting percentage, number of roots, and root length were measured after 3 weeks on the rooting medium. All data were analyzed by ANOVA, followed by Duncan's multiple range test at $P < 0.05$ by STATISTICA 10.0 (StatSoft, Inc., Tulsa, OK, USA).

3. Results and discussion

Rapid and continuous introduction of new genetic diversity into rootstock breeding is essential for selection success and efficiency. The standard procedure of aseptic culture establishment includes mercuric chloride surface disinfection, as suggested by Sedlak et al. (2008), Scaltsoyiannes et al. (2009), and Bošnjaković et al. (2013). In our study, increasing explant size and excision proximal to bud burst resulted in a higher contamination rate. Sterilization with removed outer scales and isolation of meristems with 2–3 primordial leaves considerably diminished the contamination. Meristem culture was the most suitable explant for cultures initiated from field-growing mother trees for *Prunus* species in general (Druart, 2013). The lowest contamination rate (up to 27%) was observed on dormant buds collected in November and December (Table 1). In January the contamination rate increased, while in February and March the contamination rate was over 60% in 'OV 14' and 'OV 32' genotypes. The highest contamination rate recorded for genotype 'OV 14' occurred when actively growing shoot tips taken in April were used as an explant source.

The percentage of undeveloped explants ranged from 5.3% to 69.5%, except in February, when it reached 96.6%. Rosette initiation from dormant buds was the highest (up to 87.5%) in November with 'OV 14' genotype and the lowest in selection 'OV 32' in February (9.1%). Significant improvement in culture initiation was observed in April, reaching 96.8% in 'OV 17' genotype, when shoot tips were used as an explant source.

Gisela 5 low-vigorous cherry rootstock is mainly propagated by micropropagation throughout the year (Ružić and Cerović, 2002; Ružić and Cerović, 2003). Bošnjaković et al. (2013) and Dorić et al. (2014) reported successful micropropagation when dormant buds, rather than actively growing shoot tips, were used as an explant source in *P. fruticosa* Pall. and *P. mahaleb* L., regardless of genotype diversity. Such an approach allowed fast clonal multiplication of diverse germplasm from in situ plants in the winter, whereby compatibility in the nursery could be performed the following spring.

Explant type defined initial length and shoot elongation in subsequent subcultures, as presented in Table 2. On the same initiation medium, dormant buds developed slower than actively growing shoot tips. After three subcultures most developed rosettes were with insufficient shoot elongation, ranging from 0.5 to 1.1 cm. The most significant shoot development was noted in the first and second subculture in 'OV 17' collected in March; this failed to develop further during the third subculture. When cultures were established in April the same genotype exhibited better development and achieved adequate explant length for the multiplication phase.

Table 1. Effect of explants collection date on aseptic culture establishment of *P. cerasus* L., 'Oblačinska' sour cherry accessions.

Genotype	Season explants taken	Rosette initiation (%)	Undeveloped explants (%)	Contamination (%)
'OV 14'	XI	87.5c	0.0k	12.5o
	XII	29.5o	58.8c	11.7o
	I	45.1k	18.4h	36.5h
	II	16.3r	16.4i	67.3b
	III	56.5h	0.0k	43.5f
	IV	0.0t	0.0k	100a
'OV 15'	XI	75.0d	0.0k	25.0l
	XII	43.1l	36.8f	20.1n
	I	56.3h	0.0k	43.7f
	II	0.0t	96.6a	3.4r
	III	41.0m	0.0k	59.0c
	IV	93.5b	0.0k	6.5q
'OV 17'	XI	69.7e	5.3j	25.0l
	XII	33.7n	39.6e	26.7k
	I	50.5j	19.4g	30.1j
	II	18.2q	36.3f	45.5e
	III	66.9f	0.0k	33.1i
	IV	96.8a	0.0k	3.2r
'OV 32'	XI	24.6p	50.0d	25.4l
	XII	50.5j	39.0e	10.5p
	I	61.3g	0.0k	38.7g
	II	9.1s	69.5b	21.4m
	III	18.0q	21.1g	60.9c
	IV	53.5i	0.0k	46.5d

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

After establishing *P. cerasus* L. 'Oblačinska' accessions in vitro from dormant buds, cessation of explant growth was noted in the next two subcultures in all genotypes, possibly due to accumulation of growth-inhibitor hormones. After a period of intensive growth at the end of vegetation, levels of the growth-inhibitor plant hormone abscisic acid increase, while levels of cytokinins and gibberelic acid (plant growth promoters) decline in buds (Pruski et al., 2005). Once tree dormancy commences, a period of low temperature is required to break this dormancy. All attempts to delay dormancy with hormones were unsuccessful (Borkowska, 1986). To break dormancy, Druart (2013) recommended cold storage of twigs for 1 month at 2 °C. In *P. fruticosa* and *P. mahaleb*, dormancy did not adversely affect culture initiation, multiplication, shoot elongation, or rooting (Bošnjaković et al., 2013; Dorić et al., 2014).

Explant elongation, and the subsequent multiplication phase, was observed only when aseptic cultures were initiated from shoot tips collected in April (Table 3). The index of multiplication was the highest in genotype 'OV 32', although it was generally still low (not exceeding 1.9) (Figure 1). There was no significant difference among the 'OV 15', 'OV 17', and 'OV 32' genotype shoot lengths in the first subculture. On the same medium and plant growth regulator composition, an increase in index of

Table 2. Explant length during three subcultures on different dates of explant collection.

Genotype	Season explants taken	Length of established explants (cm)	Explant length in first subculture (cm)	Explant length in second subculture (cm)	Explant length in third subculture (cm)	Explant length average (cm)
'OV 14'	XI	0.2g	1.0efg	1.0f	1.1cd	1.0ef
'OV 15'		0.3defg	1.1def	1.1ef	1.1cd	1.1e
'OV 17'		0.2efg	0.9fgh	1.0f	1.0de	0.9g
'OV 32'		0.2g	0.5jk	0.6hi	0.7gh	0.6j
'OV 14'	XII	0.2efg	0.7ghijk	1.0f	1.1cd	0.9g
'OV 15'		0.2fg	0.5jk	0.7ghi	0.8efg	0.7hi
'OV 17'		0.1g	0.5jk	0.7ghi	0.9def	0.7hi
'OV 32'		0.3def	1.0egf	1.1ef	1.0de	1.0ef
'OV 14'	I	0.2efg	0.5jk	0.6hi	0.7gh	0.6j
'OV 15'		0.2fg	0.6ijk	0.7ghi	0.8fgh	0.7hi
'OV 17'		0.3defg	0.7hijk	0.8g	0.9def	0.8h
'OV 32'		0.3defg	0.5k	0.5i	0.8fgh	0.6j
'OV 14'	II	0.2defg	0.8ghij	0.8gh	0.8fgh	0.8h
'OV 15'		0.3d	0.6ijk	0.6hi	0.6h	0.6j
'OV 17'		0.3de	0.6ijk	0.6hi	0.6h	0.6j
'OV 32'		0.2defg	0.8ghi	0.8gh	0.8fg	0.8h
'OV 14'	III	1.1b	1.4bc	1.5b	1.5b	1.5b
'OV 15'		1.0bc	1.2cde	1.2de	1.2c	1.2d
'OV 17'		1.7a	1.8a	1.8a	1.8a	1.8a
'OV 32'		0.9c	1.3cd	1.4c	1.4b	1.4bc
'OV 14'	IV	/	/	/	/	/
'OV 15'		1.1b	1.2cdef	1.3cd	1.4b	1.3cd
'OV 17'		1.6a	1.6ab	1.8a	1.8a	1.7a
'OV 32'		1.1b	1.2cde	1.3cd	1.3cd	1.3cd

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

Table 3. Multiplication parameters of in vitro shoots in two subsequent subcultures of *P. cerasus* L., 'Oblačinska' sour cherry accessions.

		First subculture		Second subculture	
Genotype	Season explants taken	Index of multiplication	Shoot length (cm)	Index of multiplication	Shoot length (cm)
'OV 15'	IV	1.0b	0.6	0c	0b
'OV 17'	IV	1.2ab	0.6	1.3b[1]	0.7a
'OV 32'	IV	1.5a	0.6	1.9a	0.6a
F test		*	Ns	*	*

* Numbers followed by a different letter are significantly different at $P < 0.05$, according to Duncan's multiple range test.
Ns: not significant at $P < 0.05$.

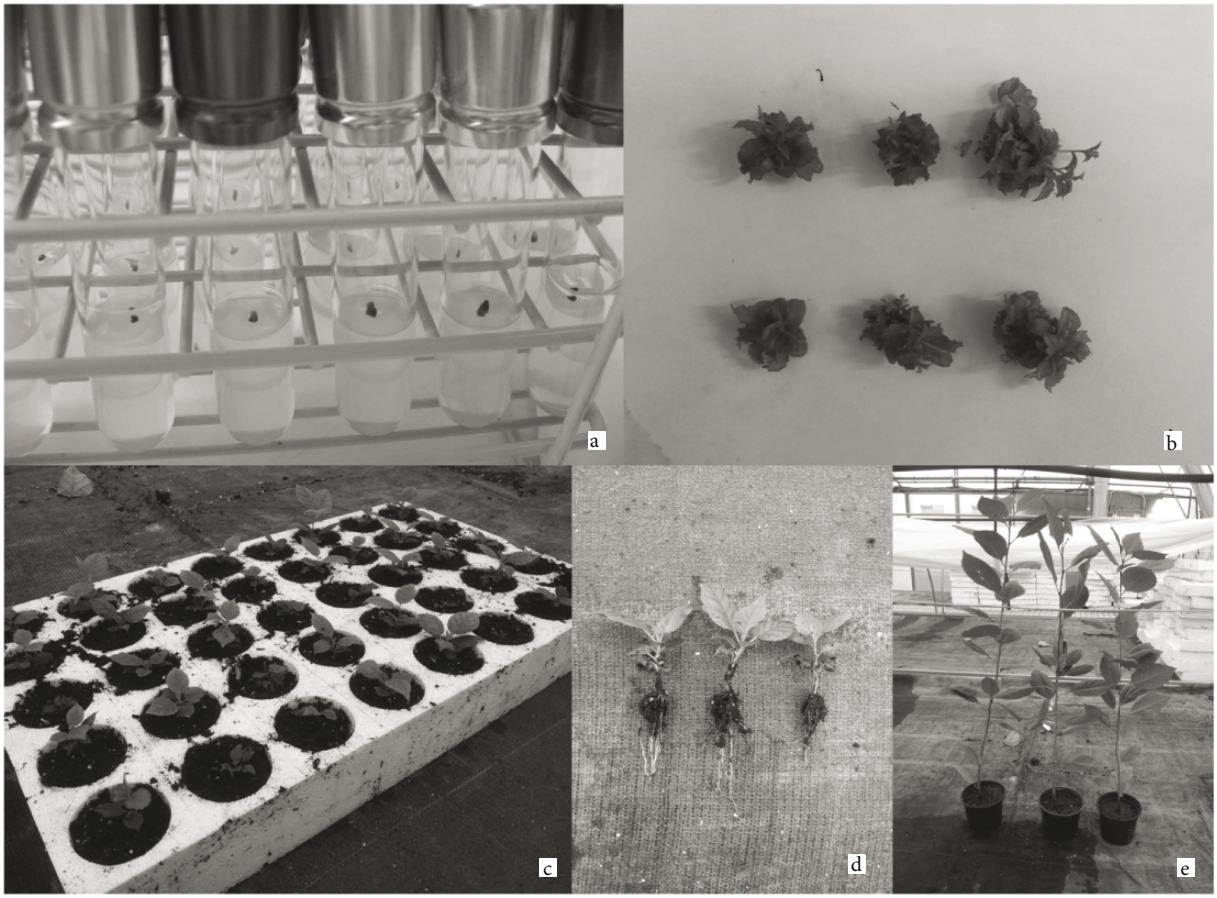

Figure 1. In vitro propagation of *P. cerasus* L., 'Oblačinska' sour cherry 'OV 32' genotype: a) establishment of aseptic culture from dormant buds; b) in vitro shoots in the multiplication stage; c, d) acclimatized plants; e) plants after 6 months growth in pots.

Table 4. In vitro rooting percentage of *P. cerasus* L., 'Oblačinska' sour cherry accessions.

Genotype	IBA concentration (mg L^{-1})	Rooting (%)	Number of roots per shoot	Total root length (cm)
'OV 17'	0.5	20.1c	2.8d	6.1c
	1.0	71.3b	6.0b	13.5a
	1.5	3.5e	1.8e	4.1d
'OV 32'	0.5	13.6d	4.4c	5.8c
	1.0	81.3a	8.1a	10.7b
	1.5	2.7e	3.3d	3.3d

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

multiplication in the second subculture was observed only in the 'OV 32' genotype.

Shoot tips obtained in April have a better survival rate and commence growth more rapidly. High levels of growth promoting substances and low growth inhibitors in actively growing shoots during early spring may be responsible for the higher index of multiplication. The index of multiplication in 'OV 32' genotype on DKW medium with 0.8 mg L^{-1} BA and 0.01 mg L^{-1} IBA was significantly lower than in *P. fruticosa* and *P. mahaleb*

selections, in which the index of multiplication exceeded 6 (Bošnjaković et al., 2013). Ružić et al. (2007) reported an index of multiplication of 1.13–1.96 for cherry rootstock Damil GM 61/1. Despite testing six different multiplication media, Sedlak and Paprštein (2013) could not exceed an index of multiplication of 1.7 in two sweet cherry cultivars 'Amid' and 'Kares Fruhe'.

Excellent rooting ability was also noted with an IBA concentration of 1 mg L^{-1}; 71.3% of 'OV 17' genotype and 81.3% of 'OV 32' genotype developed roots (Table 4). On the other hand, Singh et al. (2010) reported the maximum sour cherry rooting percentage on an IBA concentration of 2 mg L^{-1}, depending on rooting medium.

Proliferation of *Prunus mahaleb* L. could be negatively affected by *Prune dwarf virus* (PDV), as suggested by Saponari et al. (1999). All accessions used as plant material for this research have been tested for PDV. Samples were tested by DAS–ELISA method, and results were confirmed using RT PCR. PDV was detected in four samples, none of which were in *P. cerasus* L., 'Oblačinska' sour cherry accessions (Bagi et al., 2012). In 2013, PDV tests were repeated, and the results in all samples were negative.

Because of the long breeding cycle and lack of feasible conventional cloning methods, the in vitro propagation technique is a reliable approach for cloning desirable *P. cerasus* L., 'Oblačinska' sour cherry accessions. Establishing the explants in vitro in early winter is an important parameter for immediate introduction of genetic diversity into rootstock breeding procedures defined by conventional approach. An in vivo dormancy break during the winter and culture establishment by shoot tips in spring is not favorable, as it postpones the introduction of clonal material to the rootstock breeding program for one entire vegetation. Dormancy is a problem, and further investigation of ex vivo cold treatment is necessary as a precondition for micropropagation during the winter, in order to obtain clonally propagated plants in spring next year.

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