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Original scientific paper

DISTINGUISHING OBLAČINSKA SOUR CHERRY CLONES (*Prunus cerasus* L.) BY POLLEN MORPHOLOGY

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Morphology and ultrastructure of pollen grains were studied in 13 Oblačinska sour cherry clones in three years using scanning electron microscopy (SEM). All studied clones had isopolar, radially symmetric and tricolpate pollen grains. Length and width of pollen grains varied in a range of 50.03-55.97 μm and 25.16-28.37 μm respectively. Pollen shape in all the studied clones was identified as prolate. The smallest colpus length was found in the clone 8 (43.73 μm), and the highest in the clone 3 (49.16 μm). The highest colpus and mesocolpium width had the clone 3 (1.73 μm; 14.87 μm) and the lowest had the clone 7 (1.46 μm; 13.78 μm). All studied clones had striate exine ornamentation. Number of ridges per 100 μm^2 of the exine surface was the highest in the clone 11 (16.7) and lowest in the clone 2 (14.1). Ridge and furrow width ranged from 0.46 to 0.61 μm and from 0.45 to 0.59 μm, respectively. The clones have been classified into three clusters based on all studied properties. Pollen grains examination by SEM indicated that several morphological parameters (pollen size and exine characteristics) can be used to distinguish Oblačinska sour cherry clones.

Keywords: *Prunus cerasus*, clone, pollen grain, exine patern, scanning electron microscopy

INTRODUCTION

Sour cherry (*Prunus cerasus* L.) belongs to the oldest fruit trees that people use. This is confirmed by the remnants of the stones of cave people from North America and the Scandinavian Peninsula (BROWN *et al*., 1996), which indicate the existence of this species even in neolithic times (5000 years ago). It is considered that sour cherry has been obtained by

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spontaneous hybridization between the ground cherry (*Prunus fruticosa* Pall.) and wild cherry (*Prunus avium* L.). Sour cherry belongs to the *Rosaceae* family, subfamily *Prunoideae* (stone fruits), the genus *Prunus* L., the subspecies of *Cerasus* Pers. and the *Eucerasus* Koehne section (REHDER, 1974).

For centuries, people have selected and propagated by suckers or grafting sour cherry types that had good productivity and fruit quality, and at the same time they were best adapted to local environmental conditions. Thus, numerous autochthonous cherry ecotypes were created, such as: Cigány (Gypsy, Zigeuner) and Pandy (Crisana, Köröser) in Hungary, Oblačinska in Serbia, Mocanesti (Shepards) in Romania, Strauchweichseln and Weinweichseln in Germany, Stevnsbär in Denmark and Vladimirskaya in Russia (IEZZONI *et al*., 1991).

Oblačinska sour cherry, as an autochthonous variety, in sour cherry orchards in Serbia is represented with the largest number of trees. The general characteristics of Oblačinska sour cherry are: low vigour, small crown, self-fertility and high and regular productivity (GVOZDENOVIĆ, 1995). Its fruit is small (about 3 g), rounded, fairly uniform in size and ripening time. The skin is dark red and thin. Flesh is red, medium firm, juicy, rather acidic, aromatic, of good quality and suitable for processing in different products (MRATINIĆ, 2002).

MIŠIĆ (1989) stated that the existing population of Oblačinska sour cherry was mostly resulted from clonal propagation, using the suckers. However, the possibility that the generative propagation has affected the spread of this variety could not be excluded. All this conditioned that Oblačinska sour cherry today represents a heterogeneous sour cherry population, i.e. a mixture of a large number of clones - genotypes (NIKOLIĆ *et al*., 2005; RAKONJAC *et al*., 2010; FOTIRIĆ AKŠIĆ *et al*., 2014).

Since Oblačinska sour cherry is not a pure variety but a mixture of a large number of clones (genotypes), there are problems with its propagation and exploitation. For these reasons, particular emphasis should be placed on its clonal selection (NIKOLIĆ *et al*., 2005). Despite the intense work on the clonal selection, there is still no recognized clone of Oblačinska sour cherry in Serbia. This points to need for further study, selection and collecting of positive clones, for their recognition and introduction into production.

Various morphological characteristics of individual organs, such as: tree, leaf, flower and fruit, can be used to identify and characterize plant material. In addition to these, morphological traits of pollen also represent a very important parameter for the identification of certain plant species, and even some varieties within the same species (EVRENOSOĞLU and MISIRLI, 2009).

The analysis of the pollen ultrastructure was successfully used to distinguish different varieties and clones of apple (MARCUCCI *et al*., 1984; CURRIE *et al*., 1997; JONEGHANI, 2008), Japanese pear (MATSUTA et *al*., 1982), quince (NAGY-DÈRI, 2011; RADOVIĆ *et al*., 2016), walnut (LEE *et al*., 2008; MERT, 2010), pecan (WETSZTEIN and SPARKS, 1985; HAULIK and HOLTZHAUSEN, 1988), hazelnut (MIĆIĆ *et al*., 1988), chestnut (MERT and SOYLU, 2007), cornelian cherry (MERT, 2009), pistachio (DAVARYNEJAD *et al*., 1995) and olive (LANZA *et al*., 1996; LANZA and MARSILIO, 1999; JAVADY and ARZANI, 2001).

HEBDA *et al*. (1991), GILANI *et al*. (2010) and GERACI *et al*. (2012) point out that the research of morphological characteristics of pollen can be an adequate way to identify varieties in different species of the genus *Prunus*. For this purpose, the pollen ultrastructure analysis was

used in plum (MIĆIĆ, 1988), peach (RADICE *et al*., 2003; HU et *al*., 2012), apricot (ARZANI *et al*., 2005; ASMA, 2008), sweet cherry (RADIČEVIĆ *et al*., 2013; NIKOLIĆ and MILATOVIĆ, 2016) and almond (MULAS *et al*., 1988; TALAIE and IMANI, 1998; SORKHEH *et al*., 2008). Morphological characterization of pollen and determination of certain genotypes was also performed in sour cherry (NYÉKI *et al*., 1996; MIAJA *et al*., 2000; NIKOLIĆ and MILATOVIĆ, 2017).

The aim of this paper was to examine the morphology and ultrastructure of pollen of some Oblačinska sour cherry clones using scanning electron microscopy (SEM) in order to develop an identification tool for germplasm certification and for the breeding purposes.

MATERIALS AND METHODS

As the material for this study, 13 clones of Oblačinska sour cherry were used. The clones are located in the collection orchard at the Experimental farm "Radmilovac" of the Faculty of Agriculture, University of Belgrade. The orchard was planted in the spring of 1994, with a spacing of 4 × 3 m. The rootstock is the Mahaleb cherry (*Prunus mahaleb* L.), and the training system is the open vase. The investigations were conducted in the period from 2010 to 2012. During the period of study, standard cultural practices were applied, without irrigation.

For examining the morphological characteristics of the pollen grains, the flower buds in the "balloon" stage were taken from the branches into the paper bags and transferred to the laboratory. The anthers were isolated from the flower buds, placed in Petri dishes (Ø 7 cm) and dried at room temperature (24 h) until the moment of pollen release. Pollen was collected in small vials and stored at 3-5°C in a desiccator containing silica-gel until SEM analysis.

Preparation of the pollen for the analysis was carried out by mounting two-layer transparent tape on the object carrier on the microscope and applying dry pollen with a brush. Coating of pollen samples was performed with a layer of gold (20 nm thickness) using "sputter-coater" BAL-TEC SCD 005 (Capovani Brothers Inc., Scotia, NY, USA). Observation of prepared samples was performed by the scanning electron microscope (SEM) JEOL JSM-6390LV (JEOL, Tokyo, Japan) at a voltage of 15 kV. On the sample of 30 pollen grains in each clone, the following parameters were analyzed: polarity; symmetry; aperturations (number of apertures, shape of an aperture); pollen shape (polar view, equatorial view); pollen size (length, width, length/width ratio); colpus size (length, width) and mesocolpium width; characteristics of exine (ornamentation, number of ridges per 100 μm^2 of exine surface, ridge width, furrow width). The experiment was done in three replicates. In each replicate, 10 pollen grains were analyzed. Pollen grains were photographed and analyzed at a magnification of 2 000 × (whole grain) and 15 000 × (exine characteristics).

Statistical analysis was performed using two-factorial analysis of variance (ANOVA). The mean values for thirty pollen grains ± standard error were presented for each clone. The significance of differences between the mean values was determined using Tukey's test for significance at $P \leq 0.05$. Clustering of sour cherry clones into similarity groups was done using unweighted pair group average (UPGA) based on all tested characteristics. Data analysis was performed using the statistical software package STATISTICA, version 8 (StatSoft, Inc., Tulsa, OK, USA).

RESULTS AND DISCUSSION

All examined Oblačilnska sour cherry clones had isopolar and radially symmetric pollen grains with three colpate apertures. Tricolpate pollen grains were previously observed in sour cherry (NIKOLIĆ and MILATOVIĆ, 2017), although some cultivars showed tetracolporate and abnormal pollen grains (MIAJA *et al*., 2000). Pollen grains with three colpate apertures were also reported in some other species of *Prunus* genus (GILANI *et al*., 2010; GERACI *et al*., 2012). In the polar view (Figure 1a), the pollen grains of all examined clones had a round shape, while in the equatorial view (Figure 1b, 2) they were elliptical. Although the shape of pollen grains varies, in most fruit species they are spherical or oval. The shape of pollen grains in the species of *Prunus* genus is described as truncated to the elliptical in almond (SORKHEH *et al*., 2008), cylindrical in peach and plum (EVRENOSOĞLU and MISIRLI, 2009), and elliptical or obtuse-triangular in apricot (ARZANI *et al*., 2005).

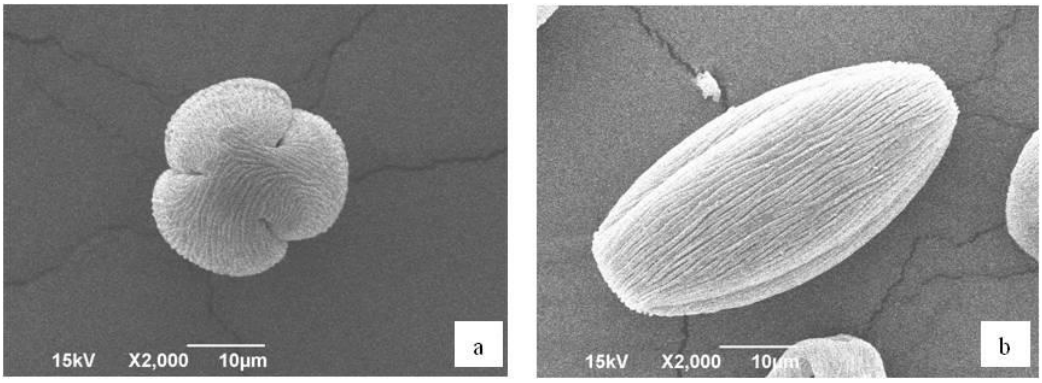

Figure 1. Pollen shape of Oblačinska sour cherry: a - polar view; b - equatorial view.

Significant differences between the studied clones were determined for the pollen length and width, while the pollen length and width ratio showed no significant variation among the studied clones (Table 1). The least average pollen length and width for all three years of testing were determined in the clone 2 (50.03 µm; 25.16 µm), and the highest in the clone 3 (55.97 µm; 28.37 µm). The average pollen length to width ratio was the smallest in the clone 12 (1.89), and the highest in the clone 2 (1.99). According to the classification of ERDTMAN (1971), pollen grains of all thirteen clones were large in size. In previous reports, pollen lenght of different sour cherry cultivars ranged from 42.5 to 48.5 µm (NYÉKI *et al*., 1996), from 41.00 to 51.61 µm (MIAJA *et al*., 2000), and from 48.0 to 52.9 µm (NIKOLIĆ and MILATOVIĆ, 2017). SÓTONYI *et al*. (2000) found slightly smaller pollen length in eight sour cherry cultivars. A similar situation is with the pollen width of different sour cherry cultivars, which in the previous work ranged from 27.5 to 30.0 µm (NYÉKI *et al*., 1996), from 23.85 to 35.10 µm (MIAJA *et al*., 2000), and from 24.3 to 27.0 µm (NIKOLIĆ and MILATOVIĆ, 2017). The average values of the pollen length and width of all examined Oblačinska sour cherry clones (51.90 µm; 26.81 µm) are

in agreement with the values of the pollen length and width (51.5 μm; 27.0 μm) that were determined for this variety by NIKOLIĆ and MILATOVIĆ (2017).

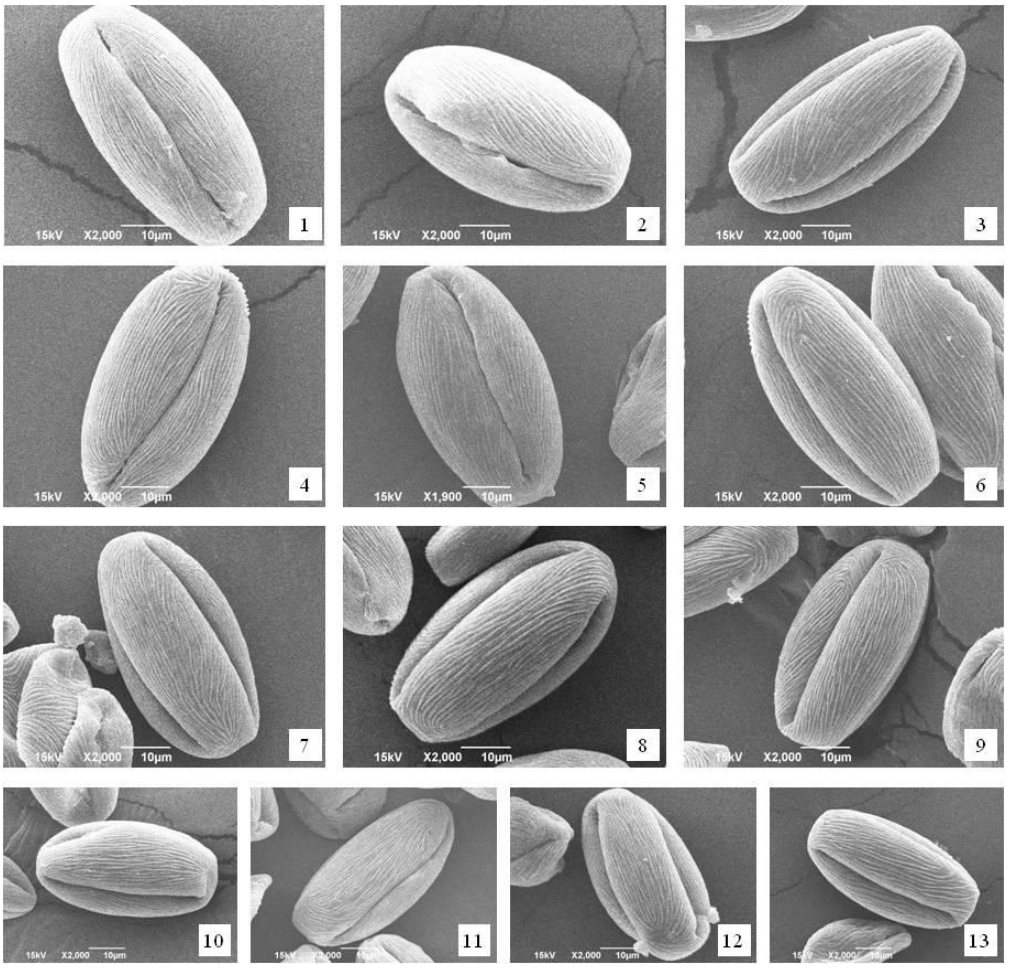

Figure 2. Scanning electron micrographs of pollen appearance in Oblačinska sour cherry (clones 1-13).

All examined Oblačinska sour cherry clones had prolate shape of pollen (L/W ratio 1.33 to 2.00) according to PUNT et al. (2007). NIKOLIĆ and MILATOVIĆ (2017) also established the prolate shape of pollen in Oblačinska sour cherry. Prolate or perprolate shape of pollen (the ratio of length and width from 1.33 to 2.00 and > 2.01) in the examined varieties of sour cherry was also determined by MIAJA *et al*. (2000). The prolate or perprolate shape of pollen was also found in apricot (ARZANI *et al*., 2005) and almond (SORKHEH *et al*., 2008), while the prolate shape of pollen was established in peach and plum (EVRENOSOĞLU and MISIRLI, 2009).

Table 1. Dimensions of pollen grains in Oblačinska sour cherry clones (average, 2010-2012).

Clone	Pollen length (μm)	Pollen width (μm)	Length/ width ratio
1	52.94±0.47 b[1]	27.37±0.23 ab	1.93±0.03 a
2	50.03±0.72 c	25.16±0.48 c	1.99±0.02 a
3	55.97±0.52 a	28.37±0.22 a	1.97±0.02 a
4	51.93±0.43 bc	26.62±0.43 abc	1.95±0.02 a
5	52.20±0.62 bc	27.23±0.31 ab	1.91±0.02 a
6	51.51±0.42 bc	26.60±0.25 abc	1.94±0.02 a
7	50.08±0.57 c	26.21±0.43 bc	1.91±0.04 a
8	50.65±0.42 bc	26.39±0.33 bc	1.92±0.02 a
9	52.85±0.25 b	27.34±0.40 ab	1.94±0.03 a
10	51.64±0.43 bc	26.47±0.21 bc	1.95±0.01 a
11	51.30±0.69 bc	26.67±0.52 abc	1.92±0.03 a
12	52.24±0.45 bc	27.57±0.35 ab	1.89±0.02 a
13	51.37±0.71 bc	26.51±0.45 bc	1.94±0.01 a
Average	51.90	26.81	1.93

[1]Mean values followed by different lower case letters in columns represent significant differences according to Tukey's test at $P \leq 0.05$.

Table 2. Dimensions of the colpus and mesocolpium in Oblačinska sour cherry clones (average, 2010-2012).

Clone	Colpus length (μm)	Colpus width (μm)	Mesocolpium width (μm)
1	46.39±0.47 ab[1]	1.55±0.06 a	14.39±0.12 b
2	43.97±0.41 b	1.72±0.06 a	14.28±0.16 b
3	49.16±0.48 a	1.73±0.05 a	14.87±0.21 a
4	45.59±0.61 b	1.64±0.05 a	14.45±0.09 b
5	45.52±0.76 b	1.57±0.05 a	14.20±0.12 b
6	44.86±0.44 b	1.62±0.05 a	14.27±0.12 b
7	43.92±0.59 b	1.46±0.05 a	13.78±0.15 b
8	43.73±0.45 b	1.55±0.06 a	13.91±0.15 b
9	46.52±0.28 ab	1.64±0.04 a	14.11±0.12 b
10	45.33±0.67 b	1.64±0.06 a	14.13±0.13 b
11	44.92±0.79 b	1.67±0.05 a	14.05±0.13 b
12	45.77±0.43 b	1.48±0.04 a	14.02±0.13 b
13	45.15±0.81 b	1.63±0.05 a	14.13±0.10 b
Average	45.45	1.61	14.20

[1]Mean values followed by different lower case letters in columns represent significant differences according to Tukey's test at $P \leq 0.05$.

Variability of the colpus length and mesocolpium width was significantly influenced by the genetically determined differences between the studied clones, while there were no significant differences for the colpus width (Table 2). Clone 3 had significantly higher average colpus length comparing to most (10) of the other clones. The largest average colpus and mesocolpium width had the clone 3 (1.73 μm; 14.87 μm), and the smallest the clone 7 (1.46 μm; 13.78 μm). The average values of the colpus length and mesocolpium width of all examined

Oblačinska sour cherry clones (45.45 μm; 14.20 μm) are in agreement with the values (45.7 μm; 14.7 μm) that were determined for this variety by NIKOLIĆ and MILATOVIĆ (2017).

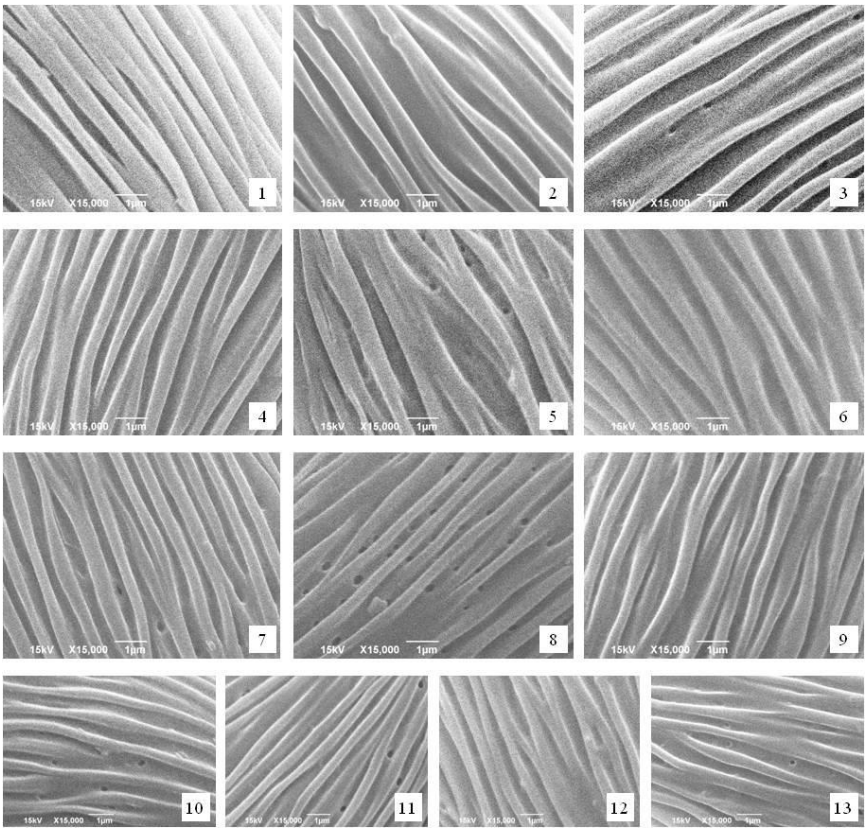

Figure 3. Scanning electron micrographs of pollen exine pattern in Oblačinska sour cherry (clones 1-13).

MIAJA *et al*. (2000) reported that the exine was thick and semitectate with vermiculate striations more or less marked in eight sour cherry cultivars. At all Oblačinska sour cherry clones examined herein, the striate exine ornamentation was determined (Figure 3). Significant presence of pits on the exine was noticed in clones 3, 5, 7, 8, 10, 11, and 13. These results are agreement with the results of NIKOLIĆ and MILATOVIĆ (2017) for the presence of pits on the exine at Oblačinska sour cherry cultivar. HEBDA and CHINNAPPA (1990) examining the pollen morphology of the *Rosaceae* family have determined that the pollen of the genus *Prunus* has a striate exine ornamentation. The presence of striate exine ornamentation in certain *Prunus* species and cultivars has been also observed by GILANI *et al*. (2010) and GERACI *et al*. (2012).

The data from Table 3 show that significant differences between the studied clones have been identified for all three exine pattern characteristics. The smallest average number of ridges per 100 μm^2 of exine surface had the clone 2 (14.1), and the highest was found in the clone 11

(16.7). NIKOLIĆ and MILATOVIĆ (2017) found that the average number of ridges per 100 μm^2 of exine surface at Oblačinska sour cherry was 16.5. In average for all three years of testing, the smallest ridge and furrow width was determined in the clone 7 (0.46 μm; 0.45 μm), and the highest in the clone 3 (0.61 μm; 0.59 μm). CHWIL (2015) found a somewhat larger furrow width, which was 0.63 μm on average for three varieties of sour cherry. In sweet cherry varieties, RADIČEVIĆ *et al*. (2013) were obtained variation intervals for the ridge width (0.36-0.40 μm) and the furrow width (0.45-0.56 μm).

Table 3. Exine pattern characteristics in Oblačinska sour cherry clones (average, 2010-2012).

Clone	No. of ridges per 100 μm^2 of exine surface	Ridge width (μm)	Furrow width (μm)
1	14.9±0.25 cde[1]	0.52±0.01 bcd	0.51±0.01 bcdef
2	14.1±0.25 e	0.55±0.01 b	0.54±0.01 abcd
3	14.3±0.22 de	0.61±0.01 a	0.59±0.01 a
4	16.0±0.22 abc	0.54±0.01 bc	0.53±0.01 abcde
5	15.1±0.29 bcde	0.54±0.01 bc	0.56±0.01 ab
6	15.5±0.28 abcd	0.50±0.01 cde	0.48±0.01 def
7	16.5±0.23 ab	0.46±0.01 e	0.45±0.01 f
8	15.4±0.28 bcd	0.54±0.01 bc	0.55±0.01 abc
9	16.4±0.17 ab	0.48±0.01 de	0.47±0.01 ef
10	15.7±0.22 abc	0.53±0.01 bc	0.52±0.01 bcde
11	16.7±0.22 a	0.51±0.01 bcd	0.47±0.02 ef
12	15.5±0.23 abcd	0.51±0.01 bcd	0.49±0.01 cdef
13	15.0±0.22 bcde	0.55±0.01 b	0.56±0.01 ab
Average	15.5	0.53	0.52

[1]Mean values followed by different lower case letters in columns represent significant differences according to Tukey's test at $P \leq 0.05$.

The dendrogram of the examined Oblačinska sour cherry clones shown in Figure 4 shows the relationship of clones to certain hierarchical levels. Based on the Euclidean distance, three groups of related genotypes were isolated. The first group consists of nine clones (1, 5, 12, 9, 4, 6, 10, 13, and 11). In the second group there are three clones (2, 7, and 8), and in the third group containes only one clone (3). The most important traits for the classification of clones in these clusters were pollen length and width and colpus length. The clone 2 from the second group with the smallest pollen length and width is associated with clones 7 and 8, which are at the same hierarchical level, and which had somewhat greater pollen length and width, but the smaller colpus length than the clone 2. For these three characteristics, clones in the first group had higher values than the previous three clones and their connection was carried out at different hierarchical levels, while the clone 3 as the only clone in the third group had the highest values for these three properties. In addition to these, other properties also had a certain influence on the classification of clones in the above-mentioned clusters. Thus, colpus width, mesocolpium width, ridge width and furrow width were smallest in the clone 7, and the number of ridges per 100 μm^2 of exine surface in the clone 2. On the other hand, the greatest colpus width, mesocolpium width, ridge width and furrow width had the clone 3.

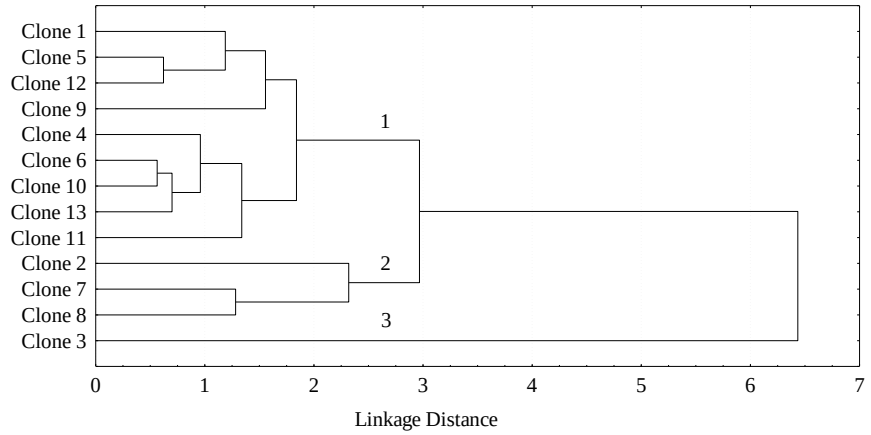

Figure 4. Dendrogram of thirteen Oblačinska sour cherry clones constructed on the basis of the Euclidean distance matrix for all studied morphological characteristics of pollen grains.

EVRENOSOĞLU and MISIRLI (2009) state that the size of the pollen grain, shape, location of the pores, their number, and exine structure are used as the most important characteristics for determination of species and varieties in fruit trees. Morphological characteristics of pollen grains and exine ornamentation were first used in the identification of species, but also varieties within the species of peach, plum, apricot, sweet cherry and sour cherry (FOGLE, 1977a, b). More recently, the determination of several genotypes of the genus *Prunus* based on the morphological characterization of pollen grains was performed in apricot (ASMA, 2008), sweet cherry (RADIČEVIĆ *et al*., 2013; NIKOLIĆ and MILATOVIĆ, 2016), sour cherry (MIAJA et *al*., 2000; NIKOLIĆ and MILATOVIĆ, 2017), almond (SORKHEH *et al*., 2008), etc. These authors generally agree on the assessment that there are differences in pollen morphology and exine ornamentation among fruit varieties, but also that these differences are often not large.

CONCLUSION

Pollen grains of all studied Oblačinska sour cherry clones were large in size with average length 51.90 μm and average width 26.81 μm. Pollen shape was prolate with length to width ratio ranged from 1.89 to 1.99. Significant differences between the tested clones were determined for all pollen and exine characteristics, except for the pollen length to width ratio and colpus width. Cluster analysis has established three groups of related clones. Among the investigated pollen characteristics, the most important for the clones distinguishing were the pollen dimensions (length and width), the colpus length, and the exine characteristics.

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RAZLIKOVANJE KLONOVA OBLAČINSKE VIŠNJE (*Prunus cerasus* L.) NA OSNOVU MORFOLOGIJE POLENA

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Izvod

Kod 13 klonova Oblačinske višnje, tokom tri godine, ispitivana je morfologija i ultrastruktura polena skenirajućom elektronskom mikroskopijom (SEM). Svi ispitivani klonovi imali su izopolarna i radijalno simetrična polenova zrna sa tri kolpatna otvora. Dužina i širina polenovih zrna varirali su od 50,03 µm do 55,97 µm, odnosno od 25,16 µm do 28,37 µm. Oblik polena svih ispitivanih klonova identifikovan je kao prolatan. Najmanja dužina kolpe bila je kod klona 8 (43,73 µm), a najveća kod klona 3 (49,16 µm). Najveću širinu kolpe i širinu mezokolpijuma imao je klon 3 (1,73 µm; 14,87 µm), a najmanju klon 7 (1,46 µm; 13,78 µm). Kod svih ispitivanih klonova utvrđena je strijatna ornamentacija egzine. Broj grebena na 100 µm^2 površine egzine bio je najveći kod klona 11 (16,7), a najmanji kod klona 2 (14,1). Širina grebena i širina brazde varirali su od 0,46 to 0,61 µm, odnosno od 0,45 to 0,59 µm. Klonovi su na osnovu svih proučavanih osobina svrstani u tri klastera. Ispitivanje polenovih zrna SEM-om pokazalo je da se nekoliko morfoloških parametara (veličina polena i karakteristike egzine) mogu koristiti za razlikovanje klonova Oblačinske višnje.

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