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RESEARCH ARTICLE

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Ovule senescence and unusual pollen tube growth in the ovary of sweet cherry as affected by pistilar genotype and temperature

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Abstract

The study of ovule senescence in the ovaries of four sweet cherry cultivars ('Karina', 'Kordia', 'Regina' and 'Summit') in the environmental conditions of West Serbia was carried out. Monitoring of ovaries was performed using the fluorescence microscopy method, on emasculated and pollinated flowers (cross-pollination variant), non-emasculated open-pollinated flowers (open pollination variant), and emasculated unpollinated flowers (unpollinated variant). In cross- and open pollination variants, the rate of unusual pollen tube growth in the ovary, fertilization percentage and fruit set were determined. The tendency in the appearance of fluorescence, as an indicator of ovule senescence, showed strong genotypic dependence – it was the most and the least pronounced in the ovaries of 'Kordia' and 'Regina', respectively, in all the flower categories. Investigation of unusual pollen tube growth, fertilization percentage and fruit set, considered from the aspect of ovule senescence and cultivars' behaviour as female (pollinated), pointed to their specific relations and complex dependence on the air temperature before and during the flowering. Flower emasculation and pollination also influenced ovule senescence, and this impact was unequal by genotypes, *i.e.* those having better ovule vitality in general, had also better ovule vitality in the conditions of emasculation, and pollination absence. The results imply different adaptation of cultivars to higher temperatures before and during the flowering, pointing to the further investigation related to the good adaptability of genotypes to air temperatures in reproductive sense, which is a basic indicator of good adaptability in general.

Additional keywords: *Prunus avium*; pistils; emasculation; pollination; fertilization percentage, fruit set.

Abbreviations used: E (emasculated); FFB (full flowering beginning); FP (fertilization percentage); FS (fruit set); NE (non-emasculated flowers); OF (ovule fluorescence); OP (open pollinated); UG (unusual pollen tubes growth in the ovary, before entrance into the micropyle); UV (ultraviolet).

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Introduction

Sweet cherry, *Prunus avium* L., is one of the economically most important members of the Rosaceae family, which is mainly grown for its fruits, suitable for fresh consumption, as well as for different kind of processing (candy and milk products, canning, juice, liqueur and jam).

Specific characteristics of the reproductive process in sweet cherries are related to some limited factors such as gametophytic self-incompatibility, flowering time and (in)sufficient flowering time overlap among compatible cultivars, fast drying of papillae on the stigma, *i.e.* short stigma receptivity, loss of ovule vitality during the flowering phenophase, the occurrence of chaotic growth of pollen tubes in the ovary, as well as the features of post-fertilization process. All of these factors, individually and collectively, can affect the fertilization success, and fruit set as its endpoint.

The ovaries in the *Prunus* species are monocarpellar, containing two anatropous ovules, one of which can be fertilized and given seed (primary ovule), whereas the other (secondary) degenerated. Ovule and embryo sac (megagametophyte) development at the moment of

flower opening, as well as their vitality during the full-flowering phenophase, are significant factors for the regular process of fertilization. The occurrence of callose is the first visible symptom of ovules degeneration in the *Prunus* species – cherries (Stösser & Anvari, 1982), almond (Pimienta & Polito, 1982) and peach (Arbeloa & Herrero, 1985). The callose started to accumulate in the chalazal part of the ovules, spreading through the integument, with the whole ovule affected at the end. The occurrence of callose can be identified by aniline-blue staining and observation under UV light. It has been found that callose accumulation is closely related to the spending of starch reserves (Rodrigo & Herrero, 1998), whose level decreases after fertilization, and plays an important role in the embryo sac nutrition (Arbeloa & Herrero, 1991). According to Pimienta & Polito (1982), deposition of callose starts before the occurrence of morphological symptoms of tissue degeneration, acting as a barrier to the translocation of the metabolites into the nucellus, stopping the growth of the ovules, and conditioning the cessation of their function. Although the function of callose has been interpreted traditionally to isolate dying from living cells, callose might also inhibit sugar transport to the aborting embryo sac (Sun *et al.*, 2004). It is not known whether this is a cause or a consequence of female gametophyte degeneration, and also the underlying molecular mechanisms of ovule abortion under temperature stress are still unknown (Hedhly, 2011). Genotype specificities and variability in terms of ovule function have been observed in plum (Cerović *et al.*, 2000), almond (Egea & Burgos, 2000), apricot (Alburquerque *et al.*, 2002), and Japanese plum (Ruiz *et al.*, 2010).

Ovary tissues provide various types of signals that orient and guide pollen tubes (Arbeloa & Herrero, 1987; Hegashiyama *et al.*, 2001; Herrero, 2001). Loss of pollen tube orientation in the ovary has been described as irregular or chaotic ("specific") growth. Earlier explanations of wandering and branching of pollen tubes are mainly associated with the occurrence of incompatibility in the ovary (Seavey & Bawa, 1986). However, Herrero (2000) stated that, even in the cases of compatible pollination, such behaviour of pollen tubes was observed; that is related to the stage of development of female flower elements, which at the time of pollen tubes arrival in certain parts of the ovary, are unable to provide their further passage. Within the temperate continental fruit species, chaotic pollen tube growth has been observed in peach (Arbeloa & Herrero, 1987), sour cherry (Cerović, 1996), plum (Đorđević *et al.*, 2010), sweet cherry (Radičević *et al.*, 2016) and quince (Radović *et al.*, 2017).

On the other hand, air temperature is the most important environmental factor that influences ovule longevity in cherries (Stösser & Anvary, 1982; Postweiler *et al.*, 1985; Cerovic & Ruzic, 1992a; Hedhly *et al.*, 2007). Air temperature also influences pollen tube kinetics and dynamics (Hedhly *et al.*, 2004); genotypic specificities in the behaviour of cultivars as pollenizers and their specific interactions with the air temperature during flowering in sweet cherries have also been shown (Hedhly *et al.*, 2005; Radičević *et al.*, 2016). According to Hedhly *et al.* (2008), incidence of seasons with high temperatures during flowering reduces fertility of cultivated plants, due to sensitivity of different phases of the reproductive process to higher temperatures. This phenomenon results in gradual changes in distribution of cultivars, favouring those better adapted in reproductive behaviour to certain conditions of air temperatures.

Investigation into the reproductive behaviour specificities of sweet cherry genotypes is the basis for understanding their adaption to different environmental conditions, particularly in the context of increasing incidence of seasons with higher temperatures during the flowering in major fruit growing regions in Europe as well as in the Republic of Serbia, by which the flowering phenology in sweet cherries has been influenced in the last decades (Wenden *et al.*, 2016), as a consequence of global warming. This viewpoint could be important for defining the goals within the sweet cherry breeding programmes, for developing cultivars better adapted to environmental condition in reproductive sense.

The investigation presented in this paper is aimed to determinate genotypic specificities in ovule senescence manifestation, the relation between the ovule senescence and characteristics of pollen tube growth in the ovary and, finally, its impact on the success of fertilization process (seen from the viewpoint of the vitality of female parts of a flower) in four world-wide commercially important sweet cherry cultivars ('Karina', 'Kordia', 'Regina' and 'Summit') grown in West Serbia conditions.

Material and methods

Plant material, growing conditions and experiment design

The experiment was conducted over two years (2009–2010) in a sweet cherry orchard at 'Preljina' facility of the Fruit Research Institute, Čačak, West Serbia (43°53' N; 20°21' E; 350 m above the sea). The average annual temperature of the area is 10.85°C, the average temperature over growing period (April–October) is 16.8°C, and total annual rainfall rate is 748.4 mm.

Four sweet cherry cultivars ('Karina', 'Kordia', 'Regina' and 'Summit') were used in the study. *S-RNase*-based genotyping using consensus and allele-specific primers gave patterns consistent with S_3S_4, S_3S_6, S_1S_3, and S_1S_2 genotypes, respectively (Radičević *et al.*, 2013), which were in agreement with results reported by Schuster *et al.* (2007) and Schuster (2012).

The cultivars were grafted on Gisela 5 rootstock, and orchard was established with two-year-old "knip" nursery-trees in the spring of 2005. The spacing of 4.0 × 1.5 m and 'Zahn Spindle' training system were used. Standard cultural practices (pruning, fertilization, drip irrigation, pest and disease control) within orchard management were included. The experiment was set up as a randomized block design (three replications with three trees each).

Air temperature before and during the flowering phenophase

The air temperature in the orchard was continuously monitored, before and during the flowering phenophase. The average mean daily temperatures and the average maximum daily temperatures of full flowering (from the date of full flowering until the tenth day thereafter), as well as the average maximum daily temperatures ten days before full flowering, were calculated for each cultivar. The beginning of full flowering was marked as a day when 80% of flowers were opened.

Pollination procedure

Two-year old branches with a synchronized population of 80–100 flowers at late balloon stage were chosen. About 2.000 flowers per cultivar were selected on this way, each replication involving 600–700 flowers from all sides of three cherry trees. The single-pistil flowers were emasculated and protected with paper bags. In the same manner, 600 flowers per cultivar were chosen for the open-pollination variant (without emasculation and bagging). The anthers at late balloon stage of each cultivar were collected and allowed to dehisce for 24–48 h at 20ºC.

At the beginning of full flowering, when stigmatic secretion was evident, pollination of emasculated flowers was done, with two touches of stigma, which ensured approximately equal amounts of pollen (Winsor & Stephenson, 1995). Some 400–500 hundred flowers per cultivar were left unpollinated. Each of them was used as a pollenizer and pollinated cultivar ('Karina' × 'Kordia', 'Karina' × 'Regina', 'Karina' × 'Summit'; 'Kordia' × 'Karina', 'Kordia' × 'Regina', 'Kordia' × 'Summit'; 'Regina' × 'Karina', 'Regina' × 'Kordia', 'Regina' × 'Summit'; 'Summit' × 'Karina', 'Summit' × 'Kordia', 'Summit' × 'Regina'), which together with the open pollination ('Karina' OP, 'Kordia' OP, 'Regina' OP, 'Summit' OP) and non-pollinated variant for each cultivar, made a total of 20 treatments processed. The branches were isolated again, and protective bags were permanently removed three weeks after the pollination.

Microscopic observation of ovule fluorescence and pollen tube growth in the ovary

A total of 30 pistils of each pollinated treatment (cross- and open pollinated variant) was fixed in the day of full flowering beginning, 72, 144 and 240 h after pollination in FPA (70% ethanol, propionic acid and formaldehyde, 90:5:5 percentages by volume). In unpollinated variant, the pistils were fixed in the day of full flowering beginning, 72 and 144 h after it. The aniline blue staining was used (Preil, 1970; Kho & Baër, 1971). The styles were separated from the ovaries and removed. The ovaries were opened along the suture, and integuments of the primary ovules were cut with a razor blade longitudinally-tangentially (Cerović & Ružić, 1992b), in order to enable better observation of the ovule fluorescence and monitoring of pollen tube growth through the ovary parts.

The ovaries were observed under UV light (Olympus BX61 microscope) at magnification 100X, and analysed by AnalySIS software. In each pistil, the larger ovule, or/and the ovule at the lower level of fluorescence was considered as the primary ovule, which was mostly visible even in the day of the beginning of full flowering. Where there were no obvious visible differences between primary and secondary ovules, their length as well as the width in the widest part were measured; the ovule with larger dimensions was considered as the primary ovule. Primary ovule fluorescence was monitored (Anvari & Stösser, 1978) in pollinated (cross- and open) and unpollinated variants, and presented per cultivar as the average for three fixation terms (the day of full flowering beginning, 72 and 144 h after it). The rate of "unusual" pollen tubes growth (wandering, branching and curling up) has been presented as the average per pollinated cultivar for three fixation terms (72, 144 and 240 h after pollination; in a cross-pollination variant, an average for three pollenizers). The number of ovaries with penetration of pollen tube into the nucellus 240 h after pollination was taken as the fertilization percentage.

Fruit set

Fruit set was determined at the beginning of ripening phenophase, as the percentage of fruits per total number of pollinated flowers remaining after the final fixation.

It was calculated per each pollinated cultivar, as an average value for the three cultivars used as pollinizers.

Statistical analysis

The data obtained for primary ovules fluorescence in non-pollinated variant were statistically analysed using two-factor analysis of variance (ANOVA). The arcsine square-root data transformations were performed. The significance of differences among mean values was determined by Duncan's multiple range tests at $p \leq 0.05$. Correlation-regression analysis was done and Pearson's correlation coefficients were determined to examine the correlations among the reproductive parameters, for both cross- and open pollinated variants. SPSS statistical software package, vers 8.0 for Windows (SPSS. Inc., Chicago, IL, USA) was used.

Results

Air temperature before and during the full flowering

The average mean daily temperatures during the full flowering in 2009 and 2010 were: 14.52°C, 12.71°C, resp. ('Karina'); 14.21°C, 12.07°C, resp. ('Kordia'); 14.24°C, 12.66°C, resp. ('Regina'), and 14.21°C, 12.07°C, resp. ('Summit'); the average maximum daily temperatures were: 18.41°C, 15.45°C, resp. ('Karina'); 17.60°C, 14.52°C, resp. ('Kordia'); 17.94°C, 14.73°C, resp. ('Regina'), and 17.60°C, 14.27°C, resp. ('Summit').

In both 2009 and 2010 years, air temperatures before full flowering were higher than during full flowering. The average maximum daily temperatures were: 21.00°C, 15.64°C, resp. ('Karina'); 22.67°C, 15.89°C, resp. ('Kordia'); 20.89°C, 15.20°C, resp. ('Regina'), and 22.67°C, 15.80°C, resp. ('Summit').

In 2009, daily maximum temperatures ten days before and ten days from the beginning of full flowering were occasionally exceeding 25°C, and were not typical for the area. For the tested cultivars, the average mean daily temperature during the full flowering, the average maximum daily temperature during full flowering, and the maximum daily temperature before full flowering were approximately higher in 2009 for 1.93°C; 3.15°C and 6.18°C, respectively.

Ovule fluorescence in emasculated non-pollinated flowers

Analysing the emasculated non-pollinated cherry flowers in three fixation terms, the stages of ovules fluorescence were monitored (Fig. 1). A certain number of fluorescent ovules was observed even in the day of full flowering beginning (Table 1). The decreasing of the number of ovules without fluorescence signs until the sixth day of full flowering phenophase were observed in all the cultivars. The rate of ovules without fluorescence statistically was not influenced by cultivar only in the day of full flowering beginning, while in other terms the rate of non-fluorescent ovules was highly influenced by cultivar, year, and their interaction (Table 1). The highest percentage of non-fluorescent ovules was observed in 'Regina' in all fixation terms, while it was the lowest in 'Kordia'. Generally, it was higher in 2010 in all the cultivars.

Comparison of the ovule fluorescence rate in different flower categories

Comparing the rate of the primary ovules fluorescence in the ovaries of examined sweet cherry cultivars, the distinctive arrangement of different categories of flowers has been determined (Fig. 2). The largest representation of ovules with signs of fluorescence was observed in emasculated non-pollinated flowers. Their representation was less in emasculated hand-pollinated

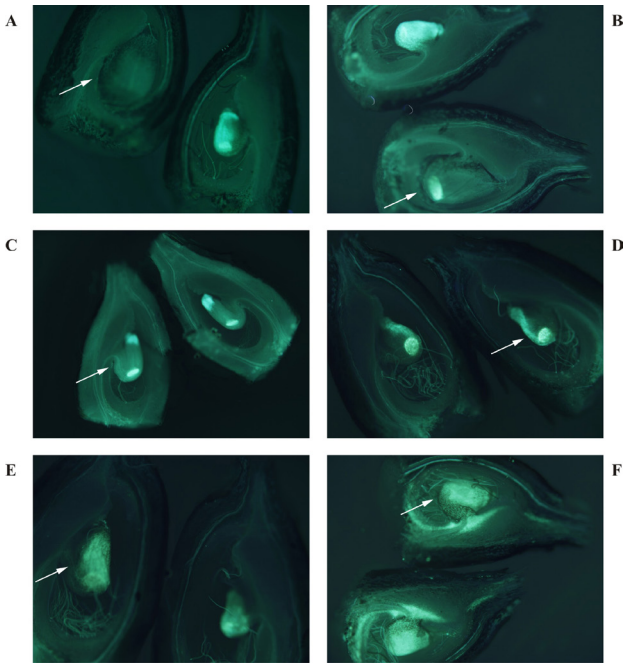

Figure 1. The stages of primary ovule fluorescence (marked with an arrow) in emasculated unpollinated flowers of sweet cherry: absence of fluorescence (A); fluorescence of chalazal region (B); fluorescence of chalazal and micropylar region (C); lateral fluorescence ("half-moon") (D); fluorescence of the entire ovule (E); fluorescence of the entire ovule, accompanied by fluorescence of surrounding tissue (F).

Table 1. Percentage of ovaries with non-fluorescent primary ovules in emasculated unpollinated flowers of sweet cherry cultivars.

Factor			The ovaries with non-fluorescent primary ovules (%)		
			The day of FFB	3rd day after FFB	6th day after FFB
Cultivar (A)	'Karina'		90.83±4.86 a	64.18±10.68 b	53.85±7.53 b
	'Kordia'		85.65±6.05 a	35.00±3.58 c	34.86±2.56 c
	'Regina'		95.00±4.86 a	81.67±7.11 a	70.46±6.74 a
	'Summit'		84.35±6.35 a	68.33±3.37 b	53.59±3.26 b
Year (B)	2009		82.48±3.88 b	47.14±3.57 b	39.04±2.55 b
	2010		95.44±2.87 a	77.46±5.62 a	67.34±4.24 a
A × B	'Karina'	2009	89.17±8.06 ab	35.76±1.04 de	26.67±0.62 d
		2010	92.50±6.56 ab	92.59±9.37 a	81.02±2.47 ab
	'Kordia'	2009	75.00±1.92 b	22.78±0.97 e	21.39±2.22 d
		2010	96.30±6.49 a	47.23±2.81 cd	43.33±0.97 cd
	'Regina'	2009	93.33±8.85 a	66.67±2.01 bc	56.67±3.93 c
		2010	96.67±6.15 a	96.67±6.15 a	84.26±9.70 a
	'Summit'	2009	72.41±1.11 b	63.33±5.45 c	46.43±1.00 cd
		2010	96.30±6.49 a	73.33±4.22 b	60.74±5.82 bc
ANOVA					
A			ns	**	**
B			**	**	**
A × B			ns	**	*

FFB: full flowering beginning. *,** and ns indicate the level of significance at $p \leq 0.05$ and the absence of significance, respectively, according to Duncan's multiple range test. Mean values followed by different lower case letters in columns represent significant differences.

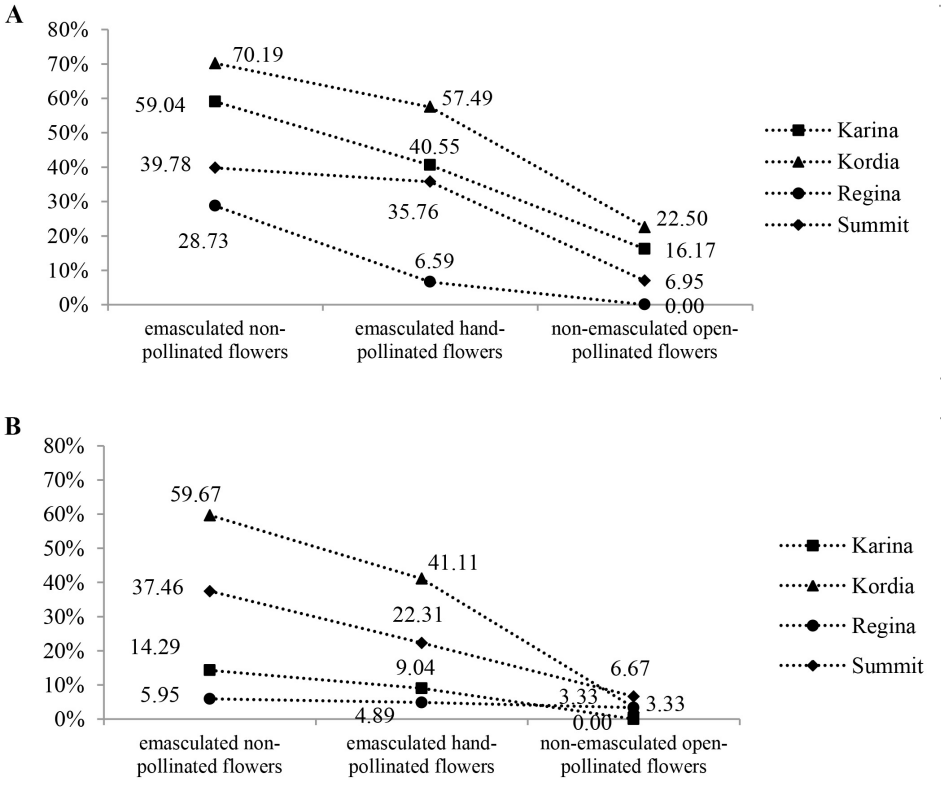

Figure 2. Percentage of primary ovules fluorescence in different categories of sweet cherry flowers in 2009 (A) and 2010 (B).

flowers (cross-pollination variant), and the lowest in non-emasculated open pollinated flowers (open pollination variant).

The highest rate of fluorescent primary ovules in all the flower categories was determined in 'Kordia' (70.19%; 57.49% and 22.50% in 2009; 59.67%; 41.11% and 3.33% in 2010, respectively), whereas it was the lowest in 'Regina' (28.73%; 6.59% and 0.00% in 2009; 5.95%; 4.89% and 3.33% in 2010, respectively).

The rate of fluorescent ovules was generally larger in 2009 for all the cultivars, and all the flower categories. The differences by years were the highest for 'Karina', and the lowest for 'Summit'.

Unusual pollen tubes growth in the ovary

By monitoring pollen tube growth in the ovaries of the tested cultivars, the presence of those with 'unusual' growth, *i.e.* the growth that deviate from 'normal', has been observed. Before the penetration into the nucellus, these pollen tubes were characterized by larger or smaller branching and the formation of bundles in the obturator zone (Fig. 3A), reverse growth in the obturator zone (Fig. 3B), the penetration of two or more pollen tubes in the micropyle (Fig. 3C), the formation of bundles in the micropyle and above the nucellar cap (Fig. 3D). In the micropyle, pollen tubes formed the bundle above and in the hatch of egzostoma, and above nucellar cap. In a few cases, thickening of pollen tubes at their tips and bending at the angle of 180 has been observed. Unusual pollen tubes growth was also identified in the nucellus, as the presence of two pollen tubes, one of which has usually been thickened (Fig. 3E), or bundle-forming, which fulfils the embryo sac accompanied by fluorescence in the embryo sac area (Fig. 3F).

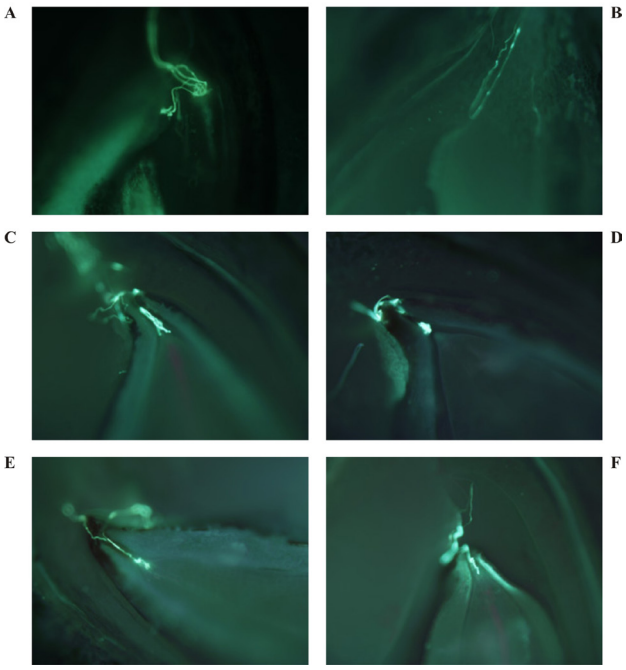

Figure 3. Unusual pollen tubes growth in sweet cherry ovaries: a bundle of pollen tubes in the obturator zone (A); reverse growth of pollen tubes in obturator zone (B); the penetration of three pollen tubes into the mycropile (C); the bundle of pollen tube above the nucellus (D); penetration of two pollen tubes into the nucellus (E); the bundle of pollen tube in the nucellus (F).

All characteristics of unusual pollen tube growth observed before the entrance into the nucellus, were also observed after the penetration (subsequently arrived pollen tubes). Different forms of unusual pollen tube growth sometimes manifested individually, or several forms manifested simultaneously, in different regions of the ovary.

Unusual growth of pollen tubes in the obturator zone before further penetration, in emasculated flowers, was the most pronounced in ovaries of 'Kordia' (33.75%), and the least expressed in ovaries of 'Karina' (13.60%) (Table 2). In non-emasculated flowers, it was also the most pronounced in ovaries of 'Kordia' (18.15%), and the least expressed in ovaries of 'Regina' (8.86%). In most cases it has been accompanied by weak or intensive fluorescence of the obturator, which could be seen even in the third, but in particular, the sixth and tenth day of full flowering.

The occurrence of unusual growth of later arrived pollen tubes was generally less frequent than before penetration into the nucellus. In the obturator zone, it was the most pronounced in the ovaries of 'Karina' (8.74% and 8.44% in emasculated and non-emasculated flowers, respectively).

Unusual behaviour of growing pollen tubes in the obturator zone in both emasculated and non-emasculated flowers was more pronounced in 2009 in all the cultivars, except in 'Regina'. In 'Kordia', it was represented in 40.18% of ovaries in emasculated flowers (Table 2).

Fertilization percentage and fruit set

In cross-pollination variant, fertilization percentage and fruit set had considerably lower value in 2009 in 'Karina' and 'Kordia', while the differences among years were small in 'Regina' and 'Summit' (Fig. 4A). The lowest value of fertilization percentage and fruit set was in 'Kordia' (3.39%; 2.50%, respectively), and the highest in 'Regina' (41.00%; 37.1%, respectively). In open pollination variant (Fig. 4B), the values of these parameters had lower values in 2009 in all the cultivars. Fruit set had the extremely low value in 'Kordia' (1.97% in 2009).

Table 2. Percentage of the unusual pollen tube growth in the ovaries of sweet cherry cultivars, in emasculated and non-emasculated flowers (cross-pollinated and open pollinated variant).

Cultivar	Year	The region of the ovary									
		Obturator				Mycropile				Nucellus	
		before penetration		after penetration		before penetration		after penetration			
		E	NE	E	NE	E	NE	E	NE	E	NE
'Karina'	2009	16.53	13.66	7.51	8.93	0.48	0.00	2.22	1.39	0.40	3.77
	2010	10.66	8.53	9.96	7.96	1.28	1.28	0.00	0.00	1.66	1.66
	average	13.60	11.10	8.74	8.44	0.88	0.64	1.11	0.70	1.03	2.72
'Kordia'	2009	40.18	23.52	1.68	0.00	0.46	3.34	0.00	0.00	0.46	3.34
	2010	27.31	12.78	3.31	0.00	0.00	0.00	0.00	0.00	1.39	1.39
	average	33.75	18.15	2.50	0.00	0.23	1.67	0.00	0.00	0.93	2.37
'Regina'	2009	15.90	12.35	7.16	3.33	1.15	1.67	0.00	0.00	0.00	0.00
	2010	18.79	5.37	1.89	5.80	1.94	0.00	0.00	0.00	0.41	2.90
	average	17.35	8.86	4.53	4.57	1.55	0.84	0.00	0.00	0.21	1.45
'Summit'	2009	24.68	16.36	1.11	0.00	1.65	1.39	1.42	1.39	0.82	4.17
	2010	23.33	8.33	5.82	3.03	2.72	3.03	1.30	0.00	0.00	0.00
	average	24.01	12.35	3.47	1.52	2.19	2.21	1.36	0.70	0.41	2.09

E: emasculated flowers (cross-pollination variant); NE: non-emasculated flowers (open pollinated variant).

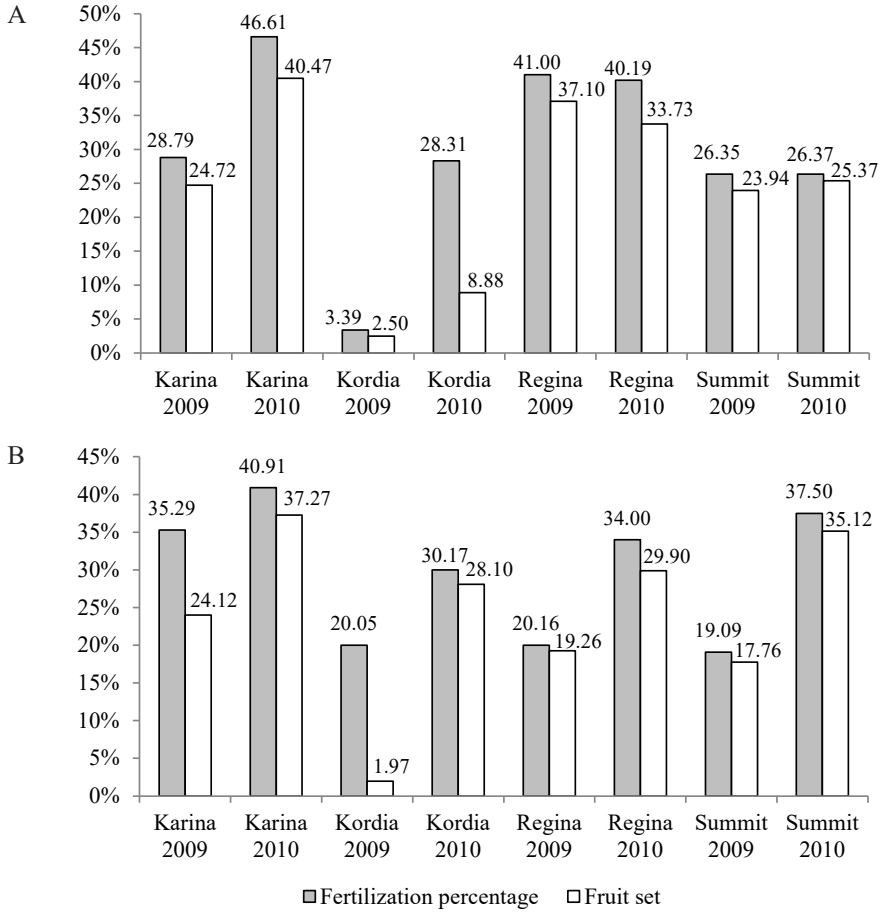

Figure 4. Fertilization percentage and fruit set of sweet cherry cultivar in cross-pollination (A) and open pollination (B) variants.

Correlation among reproductive parameters

Correlation matrix obtained for the cross-pollination variant (Table 3), showed a strong positive or negative correlation among reproductive parameters. Ovule fluorescence strongly negatively influenced fertilization percentage and fruit set (r = -0.89; r = -0.91, respectively). Similar results were obtained for the influence of unusual pollen tube growth on the parameters of fertilization efficacy (r = -0.94; r = -0.90, respectively). On the other hand, in open pollination variant (Table 3) correlation-regression analysis showed that the observed reproductive parameters were not related in the same manner as in cross-pollination variant. Ovule fluorescence had a medium-strong influence on fruit set (r = -0.71), whereas its influence on the fertilization percentage was not confirmed.

Discussion

Ovule fluorescence as a sign of senescence

The complexity of variability factors and their interactions' impact on the ovule fluorescence in emasculated non-pollinated flowers (Table 1) could be considered in the context of overlapping effects of the following: (i) genotypic specificities in terms of vitality of female elements of flowers; (ii) ovules vitality at different cultivars was unequally influenced by higher air temperatures before and during the full flowering in 2009; (iii) effect of flower emasculation was manifested unequally in different cultivars.

Table 3. Pearson's coefficients of linear correlation between the reproductive parameters in: cross-pollination variant, and open pollination variant.

Parameter	OF	UG	FP	FS
	Cross-pollination variant			
OF	—			
UG	0.79*	—		
FP	−0.89*	−0.94*	—	
FS	−0.91*	−0.90*	0.90*	—
	Open pollination variant			
OF	—			
UG	0.13	—		
FP	−0.27	−0.07	—	
FS	−0.71*	−0.01	0.85*	—

OF: ovule fluorescence; UG: unusual pollen tubes growth in the ovary, before entrance into the micropyle; FP: fertilization percentage; FS: fruit set. *The values are statistically significant at $p \leq 0.05$.

Tendency to a rapid loss of primary ovules vitality was clearly manifested in 'Kordia', while their vitality in 'Regina' was the best; the tendency was pronounced in both years of investigation. Although all the cultivars were exposed to similar temperature conditions within each year, significantly higher maximum daily temperatures before and during full flowering in 2009 could underly the unequal reactions of certain cultivars in terms of the primary ovules vitality – the impact was the most pronounced in 'Kordia', while it was the least pronounced in 'Regina', *i.e.* the negative influence of higher temperatures was more pronounced in cultivars that otherwise are prone to rapid loss of ovule vitality. 'Summit' and 'Karina' were between previous ones in terms of the expression of this trait; the differences between them, averagely observed, were small by fixation terms, so it could be concluded that these cultivars, in respect of this feature, behaved similarly. However, a more detailed analysis revealed that differences by years were relatively high in 'Karina' (strong influence of higher air temperatures before and during the flowering in 2009), and relatively small in 'Summit' (weaker influence of higher temperatures). This points to the genotypic specificities in terms of the ovules vitality, especially in the context of temperature dependence. It is interesting that statistically significant impact of the year was observed even in the day of full flowering beginning, which points to the influence of higher temperatures before and at the beginning of full flowering on ovules vitality in sweet cherries. Genotypic specificities in terms of primary ovules vitality in emasculated unpollinated flowers in the field conditions were also observed in sour cherries (Cerović & Ružić, 1992a) and in plums (Cerović *et al.*, 2000).

Ovule fluorescence in emasculated pollinated flowers (cross-pollination variant) also showed strong genotypic dependence – it was the most pronounced in 'Kordia', then in 'Summit' and 'Karina', whereas the least pronounced was in 'Regina' (Fig. 2). Genotypic dependence and variability, in terms of this trait, has also been reported in almond (Egea & Burgos, 2000) and apricot (Alburquerque *et al.*, 2002). In non-emasculated pollinated flowers (open pollination variant), the percentage of primary ovules with signs of fluorescence was lower in all the cultivars, which can be interpreted as a positive effect of the emasculation absence. Hedhly *et al.* (2009) stated that emasculation accelerated ovule degeneration. However, in this category of flowers, percentage of ovules with signs of fluorescence was relatively high in 'Kordia' (22.50% in 2009; Fig. 2A), and could affect the success of the fertilization process, and the fruit set accordingly. The parallelism in descending order of cultivars in the expression of this trait in different flower categories

has been observed (Fig. 2), which additionally goes in favour of genotypic specificities in terms of the ovules vitality, independently of flower emasculation/ pollination treatment.

The influence of temperature before and during flowering in conjunction with flower emasculation was complex and manifested unequally by cultivars. A different order of 'Summit' and 'Karina' in the expression of this trait by years has been observed (Fig. 2), which also goes in favour of more pronounced ovule sensitivity in 'Karina' to higher temperatures. Nevertheless, the impact was most pronounced in 'Kordia', and least in 'Regina', which showed apparently reduced sensitivity to higher temperatures before and during flowering. Higher flowering temperatures accelerate ovule senescence of emasculated and pollinated flowers in sweet and sour cherry (Postweiler *et al.*, 1985; Cerović & Ružić, 1992a) and in apricot (Lillecrapp *et al.*, 1999).

Comparison of ovules vitality in pollinated and non-pollinated flowers also implies the impact of pollination on the ovules vitality. Furthermore, some opposite opinions on the impact of pollination on the primary ovules vitality are noticeable. Most of the changes observed at cytohistological level in flowers during the full flowering, takes place similarly in the unpollinated and pollinated flowers, indicating that changes in female flower elements are not conditioned by pollination or fertilization in peach (Arbeloa & Herrero, 1991) and in apricot (Rodrigo & Herrero, 1998). On the other hand, Cerović *et al.* (1999) stated that a histological analisys of the ovule tissues in sour cherry in unpollinated variant has shown that the starch grains will withdraw if pollination is absent, *i.e.* withdrawing is clearly signalled and conditioned by the absence of pollination.

Unusual pollen tube growth through the ovary tissues

In both pollination variants, high percentage of the ovaries with unusual pollen tube growth, which could affect the efficiency of progamic phase of the fertilization process has been observed, being particularly pronounced in the obturator area, before further penetration of pollen tubes towards the micropyle and nucellus (Table 2). Obturator plays a key role in directional growth of pollen tubes towards the micropyle, and further, towards the nucellus in the ovary of peach (Arbeloa & Herrero, 1987). The authors stated that secretory activity of the obturator influenced the growth of pollen tubes in this area, emphasizing the fact that secretion was not continuous and not dependent on the pollination, giving the obturator a crucial role in controlling the communication between the pistil and the ovary. In some plants, the role of the micropyle in the guidance of pollen tubes in the ovary has accentuated (Herrero & Arbeloa, 1989; Dresselhaus & Márton, 2009). Synergids also play an important role in the final stage of pollen tubes growth, spermatic cells migration, and fusion of the gametes (Russell, 1996; Hegashiyama *et al.*, 2001; Punwani & Drews, 2008).

In this research, a high value of Pearson's coefficient of linear correlation among the rate of fluorescent ovules and unusual pollen tubes growth rate in the obturator zone (before further growth towards the ovule) in cross-pollination variant (Table 3), indicates that the influence of female sporophyte on the behaviour of pollen tubes in the ovary was achieved through lesser or better vitality of primary ovules. In the open pollination variant, where the ovules vitality was generally better as a result of the absence of injury of the flower emasculation, and the pollination in natural conditions, statistical significance of the coefficient of linear correlation was not determined. Hedhly *et al.* (2009) reported that pollen tubes, which behaved similarly in the growth between the stigma and the base of the style in emasculated and non-emasculated flowers, lost their directionality near the degenerated ovule, wherein the degree of this degeneration was higher in flowers that were emasculated. The influence of higher flowering temperatures in 2009 on the rate of ovaries with unusual pollen tube growth has been observed in both pollinated flower categories, as well as in all the cultivars, in particular in 'Kordia'. The average rate of unusual pollen tube growth in this work was expressed to a greater extent than reported in other fruit species – sour cherry (Cerović, 1997), plum (Đorđević *et al.*, 2010) and quince (Radović *et al.*, 2017) in similar environmental conditions.

Unusual growth of pollen tubes that later arrived in the obturator zone (after previously entering the ovule), as well as in the micropyle and nucellus, has also been observed, but to a much lesser extent. There is not much data on the nature and impact of the obturator secretion on the growth of the pollen tubes subsequently arrived. The obturator performs the function of a "temporary bridge" that connects the style with the ovary, whereby the connection is established only during the secretory phase (Arbeloa & Herrero, 1987). If the growth of the pollen tubes occurs subsequently, when the obturator is degenerated and reduced in size, the region of the ovary is isolated again.

Efficacy of the fertilization process

Genotypic specificities expressed in terms of the ovule vitality in investigated cultivars, influenced the parameters of fertilization efficacy – fertilization

percentage and fruit set. In male sphere, genotype-specific responses have also been reported at the many stages of reproductive process, including post-anthesis level – pollen germination, pollen tube growth rate and pollen tube dynamics (Hedhly, 2011).

The influence of air temperatures on the fertilization efficacy in this research can be interpreted as an overlapping combined effect on the behaviour of pollinizers, and the behaviour of cultivars in the female sphere under these temperature conditions. Higher temperatures during flowering drastically decreased fruit set in *Prunus* species (Erez *et al.*, 1998; 2000; Hedhly *et al.*, 2007). According to Sage *et al.* (2015), higher temperatures disrupt the coordinated development of male and female organs, reduce stigmatic receptivity, stigmatic pollen germination, subsequent growth within the stigma and style as well as ovule penetration, *i.e.* have a negative effect on female reproductive tissues and the growth of pollen tubes therein. The complex effect of higher maximum daily temperatures before and during full flowering in 2009 on fertilization percentage and fruit set was evident in both cross- and open pollination variants (Figs. 4A, 4B), wherein 'Regina' and 'Summit' showed the highest stability in terms of these traits, especially in the cross-pollination variant.

Comparing fertilization percentage and fruit set in cross- and open pollination variants, an unequal complex effect of air temperature and flower emasculation on the reproductive behaviour of cultivars in terms of ovule vitality and, consequently, the efficacy of the fertilization process was observed (Fig. 4). According to Nava *et al.* (2009), genetic factors are those that have the crucial role in response of the plant to high temperatures during flowering, but the environment factors can explain differences in fruit setting among the cultivars, localities, and within the same cultivar among years. Temperature stress negatively affects different stages of reproductive development – sexual organs development, progamic phase and embryo development, and these effects largely explain erratic or low fruit set (Hedhly, 2011). This author stated that in progamic phase, temperature stress influences poor pollen adhesion and germination, altered stigmatic receptivity duration, altered pollen tube growth rate, disturbed pollen tube guidance, ovule viability and longevity, asynchronous pollen/pistil interaction. The mechanisms regulating reproductive processes during exposure to high temperatures are not clearly understood, which is alarming given their recognized importance to both current agriculture and sustainability of future agriculture in a warmed-world (Sage *et al.*, 2015). On the other hand, Hedhly *et al.* (2009) stated that flower emasculation also reduced fruit set in sweet cherry, causing the significant decline of fruits in the first 2-4 weeks in emasculated, compared to non-emasculated pollinated flowers.

Ovule fluorescence, caused by genotypic specificities and environmental characteristics during the flowering phenophase, impacted the level of penetration of pollen tubes in nucellus (correlation coefficient between these parameters was -0.89 in cross-pollination variant; Table 3). In open pollination variant, statistical significance among these parameters was not observed, possibly as a result of absence of emasculation, but also the unequal moments of pollination in natural conditions. For the same reasons, the statistical significance of correlation coefficient among the unusual pollen tube growth rate and fertilization percentage has been observed in cross-pollination variant (r=-0.94), whereas in open pollination variant, it has not. The influence of ovule fluorescence on fruit set was evident in both cross- and open pollination variants (r=-0.91 and r=-0.71, respectively). It is in accordance with the results of Stösser & Anvari (1982) and Cerović (1997), who associated poor fertility in sour cherries with poor ovule vitality. On the other hand, ovule vitality was not a limiting factor for satisfactory fruit set in plum (Cerović *et al.*, 2000) and apricot (Alburquerque *et al.*, 2002).

Our research suggests that the negative effect of higher temperatures (before and during the full flowering period in 2009) and flower emasculation were particularly pronounced in cultivars that are otherwise prone to a faster loss of ovule vitality, such as in 'Kordia'. The results are in the accordance with previous ones, according to which reproductive behaviour of 'Kordia' as a pollenizer is a reflection of its geographic origin (Radičević *et al.*, 2016). 'Kordia' is an autochthonous genotype from northern Czech Republic (Bargioni, 1996), so it is better suitable for the conditions of colder climate in reproductive behaviour. Contrary, the manifested stability in terms of ovule vitality in 'Regina', influenced relatively high values of fertilization percentage and fruit set; slightly lower values in open pollination variants (Fig. 4B) could be explained by extremely late flowering and absence of well synchronized pollenizer in time of anthesis. In the manifestation of reproductive characteristics, 'Summit' and 'Karina' are between the previous ones, wherein the reproductive behaviour of 'Summit' was more similar to 'Regina', and 'Karina' behaved similar to 'Kordia'. It is interesting that investigated cultivars as pollinated, behaved on the similar way as pollenizers (Radičević *et al.*, 2016), "preferring" the same temperature condition as pollinated and pollenizers. This complies to Hedhly *et al.* (2004), according to whom stress-temperature acts as a selective pressure mechanism, favouring genotypes that are better adapted, which can play an important

role in adapting genotypes to temperature changes and global warming.

Investigation of some aspects of sweet cherry fertilization biology related to the behaviour of genotypes as female (pollinated) – primary ovules vitality, occurrence of unusual pollen tubes growth in the ovary, and the parameters of fertilization efficacy (fertilization percentage and fruit set), arising from the female domain, pointing to the their specific relations, as well as their complex dependence on the genotype and air temperature before and during the flowering. Flower emasculation and pollination also influence the ovule vitality, but this impact is unequal by genotypes, *i.e.* those having better ovule vitality in general, also have better ovule vitality in the condition of emasculation, and in non-pollinated flower category. The results imply different adaptation of cultivars to higher temperatures during flowering, pointing to the further investigation related to a good adaptability of genotypes to air temperatures in reproductive sense, bearing in mind that it is a basic indicator of good adaptability in general. According to Hedhly (2011), species or genotypes that developed tolerance and/or avoiding strategies to temperature stress are very interesting genetic reservoirs of potential temperature-tolerance genes. Such an approach can lead to the developing of cherry breeding programmes for obtaining the genotypes better adapted to the conditions of higher temperatures during flowering, which is related to challenge of global warming. Our research implies that 'Regina' should be an important part of parental combinations in these programmes. On the other hand, practical aspects of the research are related to regionalization of commercial sweet cherry cultivars, in dependence on their better or lower adaptation to environmental conditions in terms of the functioning of female elements of the flower. In West Serbia region, 'Kordia' could be "problematic" for commercial growing, due to the short life of ovules; for good results in production, not only compatible, but also a very well time-synchronized pollenizer is needed, producing the abundance of pollen at the time of flower opening in 'Kordia', and shortly after that. Contrary, 'Regina' is well adapted to higher temperatures during flowering in terms of ovule vitality, and could be important part of cultivar composition in fruit growing area in West Serbia or similar growing conditions.

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