Flora 219 (2016) 25–34

Contents lists available at ScienceDirect

Flora

journal homepage: www.elsevier.com/locate/flora

Microsporogenesis and meiotic abnormalities in different 'Oblačinska' sour cherry (*Prunus cerasus* L.) clones

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ARTICLE INFO

Article history:
Received 25 May 2015
Accepted 23 December 2015
Edited by Alessio Papini
Available online 31 December 2015

Keywords:
Chromosomes
Cytomixis
Meiosis
Pollen germination
Prunus cerasus
Sour cherry

ABSTRACT

The Oblačinska sour cherry (*Prunus cerasus*) is a mixture of different clones with similar tree and fruit characteristics that is indigenous to and widely planted in commercial orchards in Serbia. Sour cherry, including Oblačinska sour cherry clones, exhibits irregular meiosis which may contribute to low fruit set in some selections. The goal of this study was to examine the process of microsporogenesis and to determine if meiosis and its anomalies effect the *in vitro* pollen germination and pollen tube length in four 'Oblačinska' sour cherry clones that differ in fruit set and yields. All clones displayed varying degrees of chromosomal abnormalities in all meiosis sub-phases. The abnormalities became evident from late pachytene with more than half of the pollen mother cells (PMC) showing abnormal conjugation of chromosomes in metaphase I. The lowest number of PMCs with laggards was in clone III/9 and the highest in clone XIII/1. In second division, the univalent and multivalent association was observed at metaphase-II, the lagging and stickiness in anaphase-II, and the phenomenon persisted up to the microspore stage. In all four 'Oblačinska' clones, PMCs exhibited cytomixis phenomena, however, it was only observed in the second experimental year. Cytomixis differed among the four clones but was equally frequent in all stages of meiosis. The syncytia formed, most often consisted of 2–3 PMCs that were at the same phase of meiosis, and exhibited common cytoplasm and occasionally nuclear fusion. *In vitro* pollen germination and pollen tube length significantly differed between the clones. Most probably abnormalities during meiosis, regardless of the good results of pollen germination, influenced the reduced potential clones XI/3 and XIII/1.

1. Introduction

Microsporogenesis is a genetically controlled physiological, biochemical, and morphological processes where the final product is a tetrad of haploid microspores. Numerous abnormalities of meiosis can occur in plants which can result in a loss of fertility and overall reproductive efficiency (Rai et al., 2010). The most common abnormality is irregular conjugation of chromosomes such as the occurrence of univalents in diakinesis or metaphase I. Other meiotic abnormalities observed during microsporogenesis are chromosome stickiness, mixoploidy, chromosome fragmentation and failure of cytokinesis. Some abnormalities can cause chromosome elimination during microsporogenesis (Adamowski et al., 1998), the absence of collecting bivalents on the equatorial plate at metaphase I, degeneration or spindle break down (Pagliarini, 2000).

Cytomixis is an anomaly that has been identified in plant meiosis, especially in microsporogenesis, a century ago. This phenomenon is characterized by a migration of chromatin/chromosomes between proximate meiocytes (pollen mother cells, MPC) through cytoplasmic channels or intercellular bridges. In most cases it is detectable in microsporocytes (Kumar et al., 2010) and never in megasporocytes (Mursalimov et al., 2013). Cytomixis is potentially a means of maintaining heterozygosity of gametes and an additional tool for phylogenetic evolution of the karyotype by reducing or increasing the basic set of chromosomes (Cheng et al., 1987). Thus, fertile unreduced pollen grains which are '2n' may play a vital role in the sexual polyploidization of species (Kim et al., 2009). Furthermore, cytomixis has been considered to be a characteristic of genetically unbalanced plants such as

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http://dx.doi.org/10.1016/j.flora.2015.12.009
0367-2530/

hybrids, mutants and aneuploids (Rai et al., 2010). In all studied species, number of cells where cytomixis appeared is small, but it increased the pollen sterility (Pagliarini, 2000).

Factors that influence the frequency of cytomixis is pathogen attack, temperature, the use of pesticides or antibiotics, abnormal behavior due to mutagenic agents, plant fitness, presence of a mutant gene and pollution (Mandal et al., 2013). So far, cytomixis, is a well established phenomenon reported in large array of plants (Bellucci et al., 2003; Bhat et al., 2006; Kumar et al., 2010). However, all those facts are connected with herbaceous plants, while for woody perennial plants, such as fruits, reports are scarce except for plum, peach, almond, hazelnut and mulberry (Dwivedi et al., 1988; Kostritsyna and Soldatov, 1991; Lagerstedt, 1977; Soodan and Waffai, 1987).

The 'Oblačinska' sour cherry (Prunus cerasus L.) is a mixture of different clones with similar fruit and tree characteristics which is the most widely planted cultivar in commercial Serbian orchards (Ceroviæ and Radièeviæ, 2008). Accounting for 7.7% of total fruit production, with 8.7 million trees and production of 74,656 MT in 2012, Serbia is in sixth place in the world for the sour cherry production (FAOStat, 2012). Long-term cultivation in diverse agro-ecologic conditions and the use of various types of propagation (both by suckers and by seeds) has caused the 'Oblačinska' sour cherry to become a mixture of numerous genotypes (Rakonjac et al., 2010). According to several authors, who studied 'Oblačinska' populations, the highest variability among genotypes was mainly found in pomological and technological traits, including maturing time, yield, size, and fruit quality (Rakonjac et al., 2010; Fotirić Akšić et al., 2013).

Meiotic irregularities during micro-sporogenesis that can result in different levels of infertility was previously studied by Dys (1984), Dirlewanger et al. (2007), Chudíková et al. (2012) and Iordache (2013). In cherries, varying degree of irregularities at meiosis observed in microsporogenesis has been correlated with pollen germination *in vitro* (Popovska et al., 2005). Therefore, an understanding of the microsporogenesis process in sour cherry and its relationship to pollen viability is important when choosing the most productive sour cherry cultivar for commercial production. The objective of this study was to analyze cytogenetic characteristics of meiosis in different the clones 'Oblačinska' sour cherry clones that differ in fruit set and yields and to provide insight which anomalies and in what frequencies occur. Also, the goal was to prove does anomalies have impact on *in vitro* pollen germination and pollen tube length in those clones. A more precise definition of these factors will help to assess the clone fertility and will contribute to an understanding of which clone has the highest fruit set potential.

2. Materials and methods

Twigs bearing flower buds were collected from trees of four 'Oblačinska' sour cherry clones from the Experimental Station 'Radmilovac', located 8 km North-East of Belgrade (44°45′N and 20°35′E, at 135 m altitude), which belongs to the Faculty of Agriculture, University of Belgrade. The collection orchard was established in 1993. Planting distance was 4 m × 2 m. The soil is classified as Eutric Cambisol. The trees were trained as spindle bush, under non-irrigated standard cultural practices.

After five years four of the 41 'Oblačinska' clones were chosen for analysis based on their fruit set and yields. Accessions II/2 and III/9 were distinguished by their high fruit set and high yields; while accessions XI/3 and XIII/1 exhibited the lowest values fruit set and fruit yield.

In two consecutive years (2006–2007) flower buds were successively removed over several days at the first signs of swelling (approximately a month before beginning of flowering). In 2006, the flower buds removal was done in interval between 9th and 12th March, while in 2007 during last three days of February. Temperature and rainfall oscillations before and during the experiment was showed in Fig. 1, Fig. 2, respectively.

The flower primordia were excised and fixed in the rapid fixative (absolute ethyl alcohol and glacial acid = 3:1) and held in solution for 1 day. The fixed material was then rinsed in 70% ethyl alcohol and refrigerated at +4 °C. The anthers, *i.e.* PMCs (pollen mother cells) were stained for microscopic examination with 1% aceto-carmine following the standard squash technique. The preparations with a glass cover were heated slightly over a flame to increase chromosome stainability.

The pollen used in this study was collected from 50 flowers from each clone. The flower sampling was done randomly from all cardinally-oriented branches with different directions around the canopy at the balloon stage (1–2 days before opening). Anthers were removed and stored at 20 °C for 24 h.

In vitro germination of freshly collected pollen was tested on a germination medium containing agar (0.3%) and sucrose (14%). Petri dishes were kept in the laboratory at room temperature (20 ± 2 °C) for 24 h. When the length of the pollen tube exceeded its diameter, the pollen was considered germinated. For pollen germinability at least 500 pollen grains were observed and counted, and used to calculate and germination rate, respectively.

Examination of all stages in meiosis and pollen germination was done under the Leica DM LS microscope (Leica Microsystems, Wetzlar, Germany). Pollen tube length was measured for all four genotypes using the 'Leica IM 100′ program. Tests for all traits included, for both years, were done in three replicates, where each included 150 PMCs or 200 pollen grains.

2.1. Statistic analysis

The meiosis analysis was performed in two-factorial analysis of variance (ANOVA). The significances of the individual differences for the investigated factors (clone, year and interaction clone × year) were determined using the least significant difference (LSD 0.05 = 95 % confidence). Statistical analyses were conducted using STATISTICA for Windows 6.0 (StatSoft Inc., Tulsa, Okla).

3. Results

The beginning of meiosis was clearly observable from pachytene where a netlike structure was readily discernible, with chromosomes filling the whole lumen of the nucleus (Fig. 3a). In all four clones, irregular configurations of conjugated chromosomes were apparent at diakinesis. At metaphase I (Fig. 3b) or later in metaphase II (Fig. 3c) conjugated chromosomes configurations were lined up on the equatorial plate where they could be seen even more clearly. In metaphase I the percentage of PMCs with 16 normal pairing homologus chromosomes, *i.e.* bivalents, varied between all studied clones and interaction clone × year, which was proved by ANOVA (Table 1). Clone II/2 had the highest number of PMCs with bivalents in both years (48.2% and 59.9%, respectively), while clone XIII/1 showed the lowest values (30.8% and 36.1%, respectively). PMCs with differing abnormal conjugation of chromosomes, *i.e.* the pairing configurations in the form of trivalents, quadrivalents, the absence of pairing (univalents) or even combination of several different configurations (1 univalent + 1 trivalent, 2 univalents +1 trivalent, 2 univalents + 1 quadivalent) (data not show) were observed at this stage (Fig. 3d). They occurred in statistically different numbers of PMCs. Years of study did not have any influence to bivalent formation, but showed strong effect on abnormal conjugation of homologous chromosomes (Table 1).

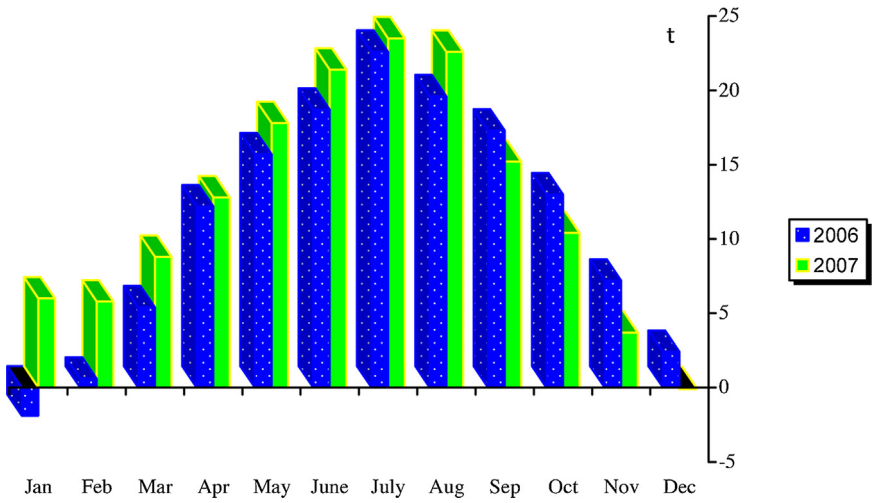

Fig. 1. Average air temperature (°C) during experiment.

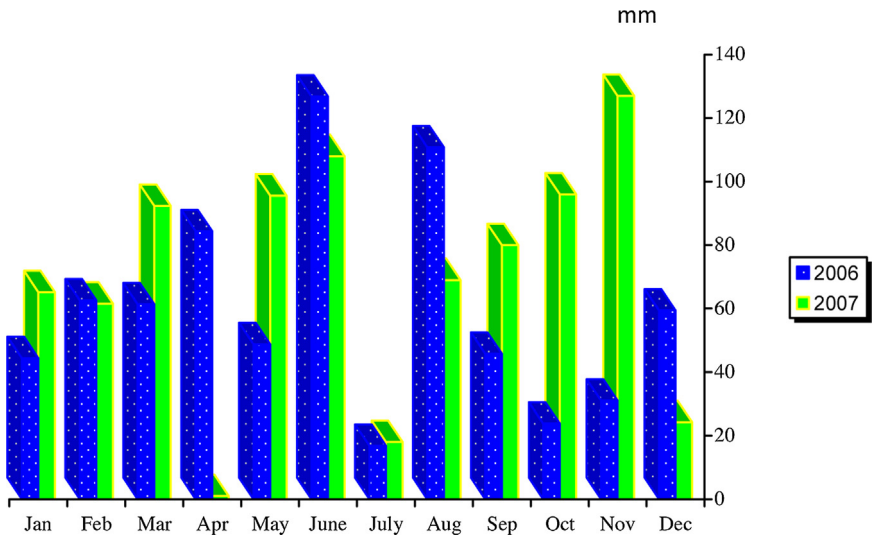

Fig. 2. Average amount of precipitation (mm) during experiment.

Table 1
Meiotic analysis of four clones of 'Oblačinska' sour cherry in metaphase I.

Factor		Percentage of PMCs with		
		Bivalents	Abnormal conjugation*	Cytomiksis
Clone*	II/2	54.0**d	42.1a	3.9a
	III/9	36.5b	57.9c	5.3b
	XI/3	44.4c	49.6b	6.0c
	XIII/1	33.4a	58.2d	7.4d
Year				
	2006	42.1	56.4b	0.5a
	2007	42.1	47.5a	10.8b
Interaction clone × year				
II/2	2006	48.2e	50.0d	1.8b
	2007	59.9f	34.1a	6.0c
III/9	2006	40.6d	57.0f	0.0a
	2007	32.5b	58.8g	10.5
XI/3	2006	48.8e	51.2e	0.0a
	2007	40.0d	48.0b	12.0d
XIII/1	2006	30.8a	67.3h	0.0a
	2007	36.1c	49.1c	14.8e

*PMCs which contains one or more univalent's, tri-and quadrivalents and also in different combinations each other.
*Significant differences are marked with different letters at $p < 0.05$.

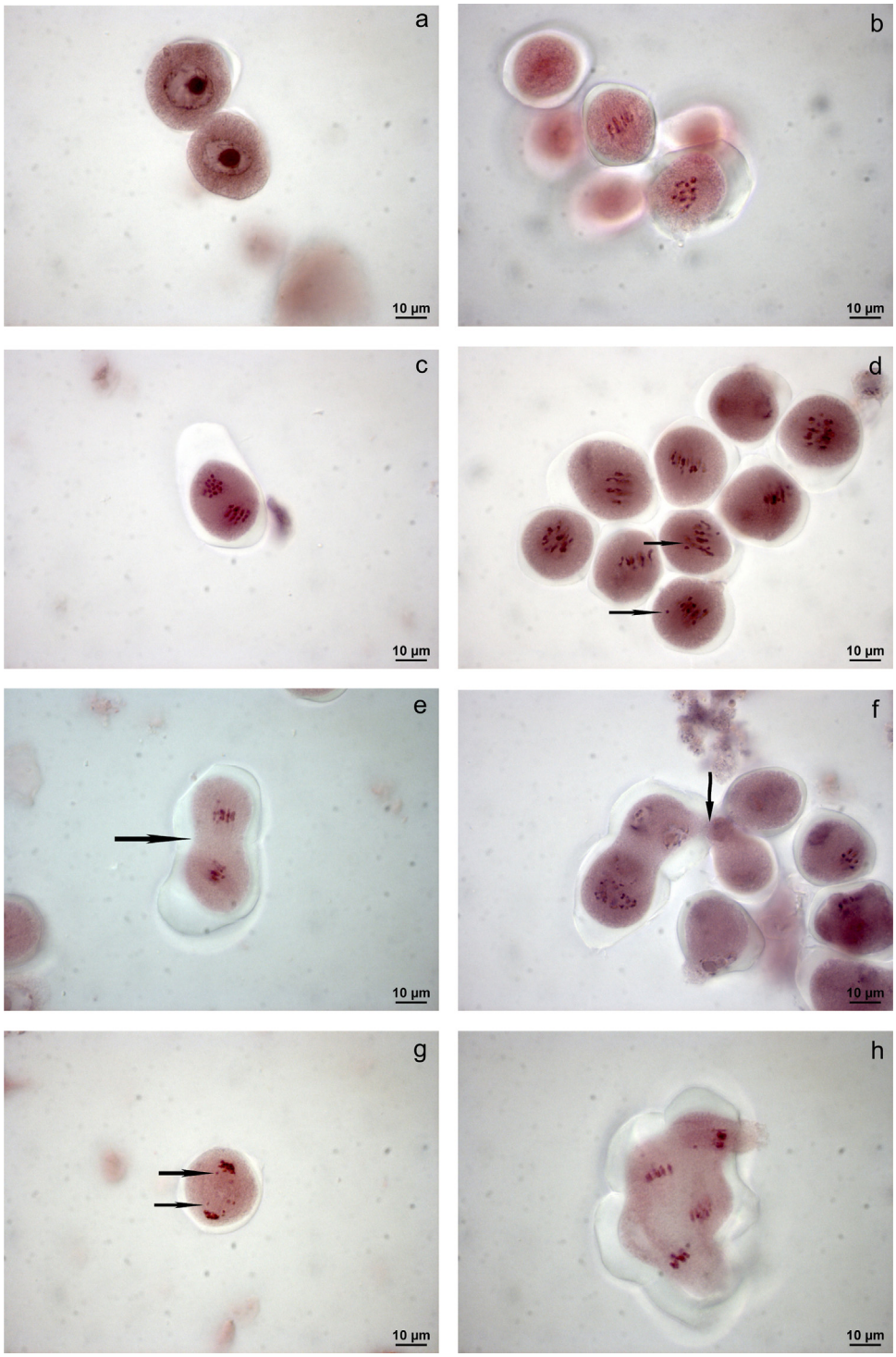

Fig. 3. Process of microsporogenesis in oblacinska sour cherry.

During these phase, cytomixis was detected in all clones, in particular in the second year of study where it varied from 6.0% in clone II/2 up to 14.8% in clone XIII/1. Only in clone II/2, was this phenomenon also observed in the first year of study (1.82%). PMCs with cytomixis were readily recognizable by their size, shape, multinuclear nature or chromosome number. In these PMCs, nuclei originating from different cells, in most cases, remained unfused and their chromosomes did not intermingle. Cytoplasmic channels between PMCs that were at the same meiotic stage were mostly broad (Fig. 3e), allowing a large fusion area, but sometimes quite narrow (Fig. 3f).

The characteristics concerning telophase I and II during the 2006 and 2007 are presented in Tables 2 and 3. In all clones, PMCs percentage with lagging chromosomes was more frequent in 2006 than in 2007. The number of lagging chromosomes ranged from one to seven (Fig. 3g). The PMCs with one lagging chromosomes were most constant (14.71%–35.34%), whereas those with several lagging chromosomes (3 or over 4) per PMCs were less persistent (0.75%–8.57%). The phenomenon called cytomixis was observed in

Table 2
Meiotic analysis of four clones 'Oblačinska' sour cherry in telophase I.

T I							
Factor		Percentage of PMCs with lagging chromosomes					Percentage PMCs with cytomixis
		0	1	2	3	Over 4	
Clone							
II/2		55.65**c	21.70b	12.26b	3.53a	2.43b	4.48a
III/9		58.99d	17.36a	11.53a	4.46c	3.10d	4.41a
XI/3		51.96b	21.62b	13.88d	4.61d	1.90a	6.04b
XIII/1		42.25a	28.21c	13.54c	4.34b	2.65c	9.01c
Year							
2006		54.30b	25.30b	13.09b	4.51b	2.74b	0.00a
2007		50.13a	19.15a	12.51a	3.96a	2.30a	11.97b
Interaction clone × year							
II/2	2006	61.29f	24.73e	11.82b	1.08a	1.08c	0.00a
	2007	50.00c	18.66b	12.69d	5.97g	3.77g	8.96c
III/9	2006	56.22e	20.00c	11.30a	6.96h	5.22h	0.00a
	2007	61.76g	14.71a	11.76b	1.96b	0.98b	8.82b
XI/3	2006	53.92d	22.55d	15.69f	4.90e	2.94e	0.00a
	2007	50.00c	20.69c	12.07c	4.31d	0.86a	12.07d
XIII/1	2006	45.76b	33.90f	13.56e	5.08f	1.70d	0.00a
	2007	38.74a	22.52d	13.51e	3.60c	3.60f	18.02e

** Significant differences are marked with different letters at $p < 0.05$.

Table 3
Meiotic analysis of four clones 'Oblačinska' sour cherry in telophase II.

Factor	Percentage of PMCs with lagging chromosomes					Percentage PMCs with cytomixis
	0	1	2	3	Over 4	
Clone						
II/2	46.82**a	29.24d	14.52c	1.40a	1.77c	6.27d
III/9	56.89d	26.51c	9.00a	3.23b	0.85b	3.54a
XI/3	50.99c	23.28a	16.08d	3.30c	2.12d	4.24b
XIII/1	49.09b	25.57b	13.85b	6.54d	0.00a	4.96c
Year						
2006	54.53b	29.12b	11.28b	3.78b	0.55a	0.75a
2007	47.36a	23.18a	15.44a	3.45a	1.82b	8.74b
Interaction clone × year						
II/2	2006	47.37c	35.34h	12.03d	0.75a	3.01b
	2007	46.26b	23.13c	17.01g	2.04b	9.52e
III/9	2006	58.22g	28.77f	8.90a	3.42e	0.00a
	2007	55.56f	24.24e	9.09b	3.03d	7.07c
XI/3	2006	62.99h	22.83b	11.81c	2.36c	0.00a
	2007	38.98a	23.73d	20.34h	4.24f	8.47d
XIII/1	2006	49.52e	29.52g	12.38e	8.57h	0.00a
	2007	48.65d	21.62a	15.31f	4.50g	9.91f

** Significant differences are marked with different letters at $p < 0.05$.

both meiosis (I and II) but only in the second experimental year. The only exception was clone II/2 where cytomixis was detected in both years. Syncyties were usually consisted of two cells (Fig. 3e), while three to five-cell sincytes were also not that rare (Fig. 3h). Fused PMCs appeared at a different frequency that varied between 8.82% (clone III/9) and 18.02% (clone XIII/1). Similar to the earlier stages, in syncyties during anaphase I and anaphase II one or more of lagging chromosomes can be found. Regarding anomalies in T I and T II, significant differences were determined between all selected clones, years of study and interaction clone x year (Tables 2 and 3).

Abnormal conjugation and segregation in all phases and subphases of microsporogenesis resulted in different frequencies of certain groups of microspores (Table 4). The number of normal tetrads in all four tested clones was significantly higher in 2006 compared to 2007. In that year, clone II/2 had the highest number of normal tetrads (69.65%), while clone XI/3 had the lowest (41.77%). During the second year, the order was different, with a very little difference between the clones in terms of normal tetrad frequencies, which varied from 29.61% (clone II/2) up to 36.47% (clone XIII/1). The other groups of microspores observed at this stage were irregular tetrads, peptads (Fig. 4a) and, in a somewhat lower number of dyads (Fig. 4b and 4c), triads (Fig. 4d), hexads (Fig. 4e), septads (Fig. 4f), octads, nonads (Fig. 4g) and decades (Fig. 4h). Irregular tetrads and pentads were more frequent in all clones as compared with other different types of anomalies.

Due to cytomixis, numerous poliads were observed. No matter the fact that syncyties were built of 2–3 or even more PMCs, they usually showed a common cytoplasm, while each genome maintained its integrity. Most likely there was just an exchange of cytoplasmic genetic material. In some cases complete genome fusion (Fig. 4i) was recorded as well. Chromatin transfer appeared to occur from cell to cell, through the narrow connection between two PMCs, and the majority of chromosomes are relocated, so microspore with extremely low cell content appeared (Fig. 4j).

Actually, in this stage, the differences between polyads incurred by cytomixis and polyads caused by chromosomes lagging in the division spindle can be observed. Polyads that underwent cytomixis have callose wall that divide the developing structure into two to three parts depending on how many PMCs were involved in the syncytia structure (Fig. 4g and h). Polyads formed from irregular chromosome divisions did not exhibit this segmentation (Fig. 4a and e).

In both experimental years the appearance of the phenomenon called 'collapsed cell' was noticed (4k). In the first year, collapsed

Table 4
Meiotic analysis of four clones 'Oblačinska' sour cherry—the tetrad phase.

Factor		Monads	Dyads	Triads	Normal Tetrads	Abnormal tetrads	Pentads	Hexads	Septads	Octads	Nonads	Decades	Collapsed cells
Clone													
II/2		1.72**c	2.62a	2.92a	49.63d	24.16d	5.04a	2.71b	1.00c	0.57c	0.00a	0.00a	9.66b
III/9		0.27a	3.75c	4.16b	46.48c	21.44c	8.02c	3.34d	1.39d	0.46b	0.46d	0.27b	9.99c
XI/3		7.97d	6.87d	5.90c	37.42a	19.54a	6.75b	2.79c	0.28a	0.25a	0.17b	0.00a	12.09d
XIII/1		0.85b	3.38b	9.04d	44.98b	19.78b	10.26d	1.93a	0.61b	0.73d	0.24c	0.00a	8.22a
Year													
2006		1.19a	3.22a	7.17b	56.00b	21.83b	5.23a	0.95a	0.18a	0.07a	0.00a	0.00a	4.17a
2007		4.21b	5.09b	3.84a	33.26a	20.63a	9.80b	4.44b	1.45b	0.93b	0.43b	0.14b	15.81b
Interaction clone × year													
II/2	2006	0a	3.52d	3.25b	69.65h	18.70b	4.07a	0.27a	0.27bc	0.27b	0.00a	0.00a	0.00a
	2007	3.43d	1.72b	2.58a	29.61a	29.61g	6.01c	5.15f	1.72e	0.86d	0.00a	0.00a	19.31g
III/9	2006	0a	2.07c	5.06d	59.08g	23.68f	5.52b	0.69b	0.23b	0.00a	0.00a	0.00a	3.68b
	2007	0.54b	5.43f	3.26b	33.88c	19.20c	10.51	5.98g	2.54f	0.91e	0.91d	0.54b	16.30e
XI/3	2006	4.76e	5.84g	7.36e	41.77e	22.29d	4.11a	0.65b	0.22b	0.00a	0.00a	0.00a	12.99d
	2007	11.18f	7.89h	4.44c	33.06b	16.78a	9.38e	4.93e	0.33c	0.49c	0.33b	0.00a	11.18c
XIII/1	2006	0a	1.45a	13.01	53.49f	22.65e	7.23d	2.17d	0.00a	0.00a	0.00a	0.00a	0.00a
	2007	1.69 c	5.31e	5.07d	36.47 d	16.91a	13.29f	1.69c	1.21d	1.45f	0.48c	0.00a	16.43f

** Significant differences are marked with different letters at $p < 0.05$.

cells were only observed in clones III/9 and XI/3. In the second, the incidence of collapsed cells ranged from 11.18% (clone XI/3) to 19.31% (clone II/2). In some cases, in such cells no reduction division occurred, and instead of creating four microspores only one monad was formed (Fig. 4l) containing the complete cell genome. Regarding anomalies in tetrad phase significant differences were determined between all selected clones, years of study and interaction clone x year (Table 4).

Examination of *in vitro* pollen germination represents the best indicator for assessing pollen viability, ie. the ability of pollen grains to perform their functions and to transfer the spermatic nucleus to the egg cell. Results of *in vitro* pollen germination for all four Oblačinska clones cherry are presented in Table 5. Pollen of clones II/2 and III/9 (Fig. 5a) had significantly higher germination rate (68.8% both clones) in both years compared to clones XI/3 and XIII/1 (Fig. 5b) (54.6 and 52.5%, respectively). Also, average values of pollen germination in all four clones was higher in 2006 (62.4%) compared to 2007 (59.7%), which was proved by ANOVA (Table 5).

Similar to pollen germination, the average length of pollen tubes (Table 5) was much different between the studied clones, and was significantly higher in clones II/2 and III/9 (1121.8 and 1064.4 μm, respectively) than in XI/3 and XIII/1 (797.7 and 752.8 μm, respectively). Besides that, ecologic factors and clone x year showed high impact on pollen tube length

4. Discussion

Microsporogenesis in all four 'Oblačinska' sour cherry clones exhibited a range of meiotic irregularities. These irregularities may be due to the polyploidy origin of sour cherry, in that sour cherry, *P. cerasus* ($2n = 4x = 32$), is a result from natural hybridization between *P. avium* ($2n = 2x = 16$) and *P. fruticosa* ($2n = 4x = 32$). Sour cherry is a segmental allotetraploid as it exhibits both disomic and tetrasomic inheritance (Beaver and Iezzoni, 1993). This genetic structure is consistent with the irregular chromosome pairings observed such as the occurrence of univalents and polyvalents, chromosomes lagging, non-disjunction of bivalents on the equatorial plane, irregular number of microspores at 'tetrads' stages and collapsed microspores, as was previously determined in other cherry cultivars and interspecific hybrids (Schuster, 2005). All of these irregularities and subsequent lagging chromosomes lead to polyads with aneuploid chromosome numbers. The presence of tetravalents during diakinesis also supports the segmental allotetraploid nature of sour cherry (Latoo et al., 2006), which is some cases uparticipated with more than 4% of the total anomalies in prophase I, and in combination with univalent with over 6% (data not show). Associated irregularities include the formation of multiple spindles and premature cytokinesis (Mendes-Bonato et al., 2003).

The high frequency of chromosomal irregularities is due at least in part to genetic effects background because each step in meiosis is genetically controlled, but it is also influenced by environmental factors (Dawe, 1998). External factors during differentiation of cherry flower buds, which occurs during the growing season prior to bloom, can significantly affect the process of microsporogenesis and pollen viability (Popovska et al., 2005). A greater number of abnormalities in 'Oblačinska' sour cherry clones were manifested in 2007 compared to 2006, with the exception of clone XIII/1 which showed a completely reversed result. For deciduous fruit trees from temperate regions such as sour cherry, the accumulation of a certain amount of low temperatures is a very important factor for further growth. Lack of chilling, associated with mild winter temperatures and drought can results in abnormal bud development (Marafon et al., 2011), and have a detrimental effect on male spore formation and pollen maturation which significantly affecting male fertility and seed set (De Storme and Geelen, 2014). Therefore, the discrepancy between the two years of this study is may be due to dry periods during autumn of 2006, when archesporial tissue in anthers starts to occur together with the differentiation of PMCs that over winter (Fig. 3). Clone XIII/1, which has the most vigour compared to the other clones, presumably due to better water supply, may have not been under water stress compared to the other clones. Extremely warm winter 2006/07 without sufficient low temperatures also would have affected the irregular winter rest period, potentially leading to a high number of anomalies (Fig. 2). In our study, average number of PMCs with anomalies in Telophase I was from 41.01% (III/9) to 57.75% (XIII/1), while in Telophase II range of variation was narrower, from 43.11% (III/9) to 53.18% (II/2). Our results are pretty higher than those obtained by Popovska et al. (2005) while studying micropsorogenesis of Oblacinska sour cherry in Macedonia. This discrepancy is probably due to different agroclimatic conditions.

In our study in metaphase I almost half of the PMCs showed abnormal conjugation of homologous chromosomes, while later in almost half of the PMCs in telophase I and II chromosome lagging was observed. In the case of some genotypes that have hybrid origin, like sour cherry is, a lack of pairing and crossing-over, yielding univalents instead of bivalents at metaphase I can be shown (De Storme and Mason, 2014). Cytological analysis revealed that these univalents either show a unidirectional segregation to one pole, or alternatively display chromosome lagging and thus remain positioned at the cell equator (Shamina et al., 2003). Besides uni-

valents and bivalents, in metaphase I the chromosomal stickiness became evident where intense clustering was observed. However, only mild occurrence of this phenomenon, where trivalents and tetravalents appeared, was detected. Chromosomal stickiness can be due to the presence of mutant genes or abiotic factors as high temperatures and herbicides (Caetano-Pereira et al., 1995). In most cases the consequence of irregular chromosomes pairing is unequal chromosome segregation and chromosome elimination, giving rise to an asymmetrical cytokinesis after meiosis (Mendes-Bonato et al., 2006). In clones II/2 and XIII/1 this phenomenon was found to be more frequent in 2006 than in 2007, in III/9 was equal in two consecutive years, while in XI/3 was more numerous in 2007, so, it is therefore concluded that chromosome stickiness in the clones might be genetically controlled.

Unbalanced segregation and lagging, led to the formation of micronuclei at Telophase I in both meiosis I and II which persisted up to the tetrad stage which resulted in pentads, hexads, septads and octads formation. On the other hand, monads, diads and triads, the structures that can be interpreted as missing meiotic division I or II, respectively, appeared in frequent number. It is proved that

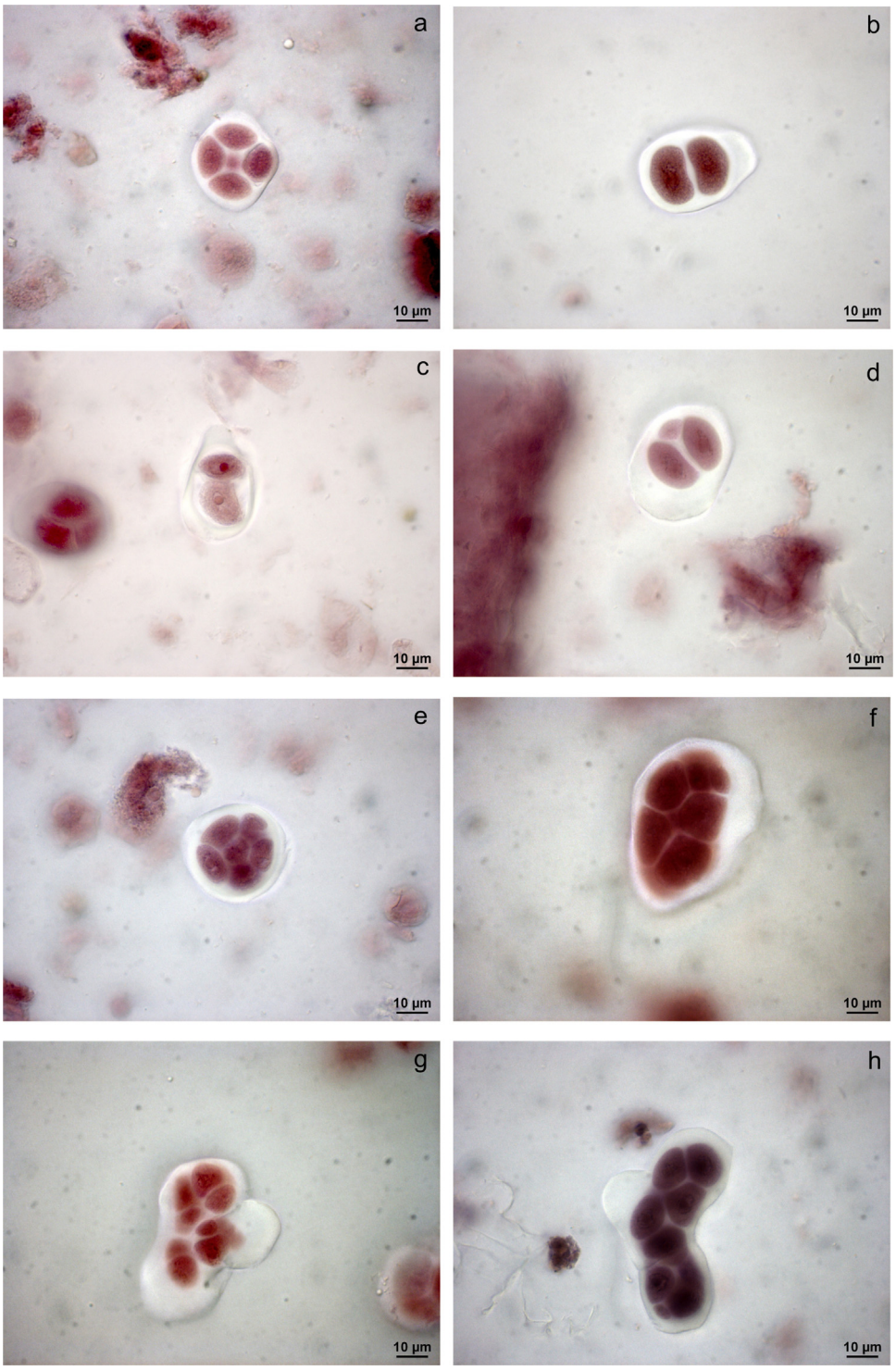

Fig. 4. The tetrad stage.

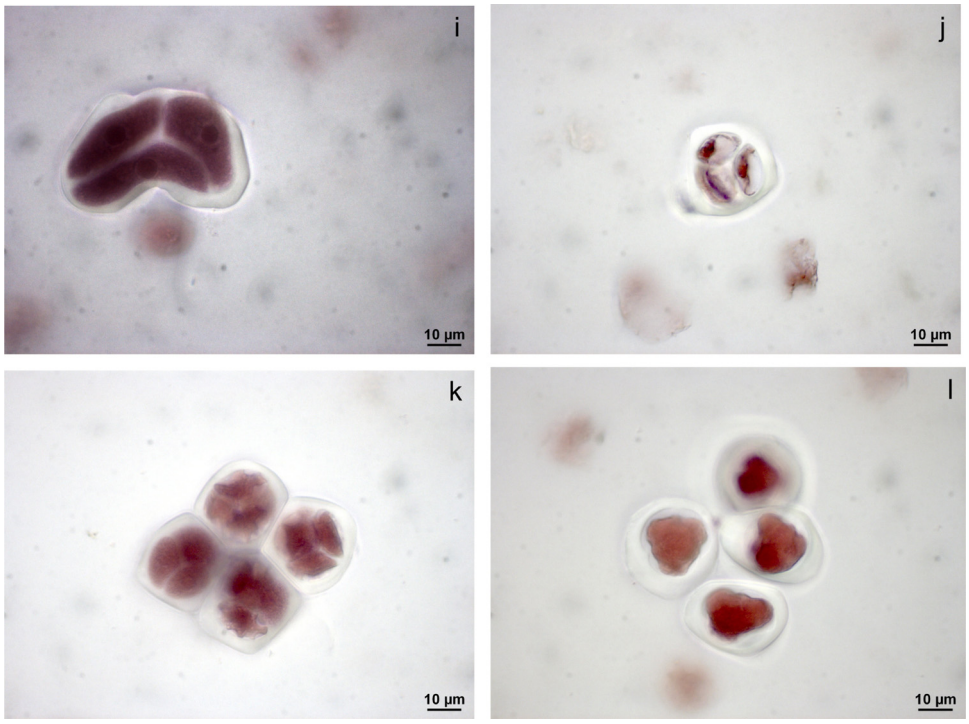

Fig. 4. (*Continued*)

Table 5
Germination of pollen grains and pollen tube length in four clones 'Oblačinska' sour cherry.

Factor		Pollen germination (%)	Pollen tube length(µm)
Clone			
II/2		68.8**c	1121.8d
III/9		68.8c	1064.4c
XI/3		54.6b	797.7b
XIII/1		52.5a	752.8a
Year			
2006		62.4b	994.4b
2007		59.7a	873.9a
Interaction clone × year			
II/2	2006	67.8e	1189.24g
	2007	69.7f	1054.3f
III/9	2006	68.8ef	1126.3 e
	2007	68.7ef	1002.4d
XI/3	2006	57.7d	862.1c
	2007	51.4b	733.2a
XIII/1	2006	55.4c	799.9b
	2007	49.6a	705.6a

** Significant differences are marked with different letters at $p < 0.05$.

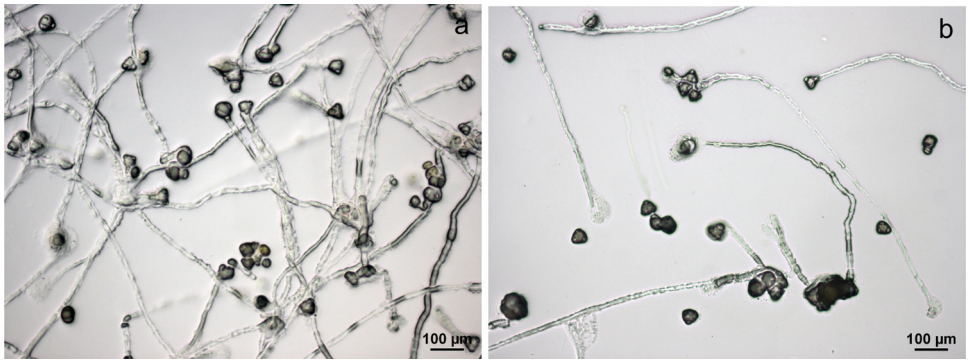

Fig. 5. Pollen germination.

environmental stresses induce or enhance meiotic non-reduction and are associated with formation of 2n gametes where Lu et al. (2013) found that pachytene to diplotene stages comprise the most optimal period for temperature stress restitution of meiosis I.

The phenomenon called 'cytomixis', which role in plant evolution is considered an additional mechanism for the origin of aneuploidy and polyploidy, is also observed in this study, which is until now rarely observed in fruits. Cytomixis has been associated with the formation of PMCs cells with an abnormal number of chromosomes or abnormal microspores (triads, pentads, septads, nonads, decads), pollen sterility, chromosomes stickiness and syncyties formation (Haglund et al., 2011). As a matter of fact PMCs which created syncytia, in all of the observed cases, were in the same stage of reduction division like Sheidai et al. (2005) noticed in pomegranate. Sometimes, in some plant species cytomixis is present in the same percentage in almost all phases during meiosis, ending with 'tetrad' stage (De Souza and Pagliarini, 1997). The occurrence of cytomixis in our study, in all tested clones, was generally between 5 and 15%, in contrast to almonds where in some individual cases cytomixis ranged from 12.5 to 97.0% of the tested PMCs (Soodan and Waffai, 1987). This discrepancy is probably due to different fruit species tested and totally different ecological conditions. However, in some plant species the number of cells where cytomixis was observed was relatively low, but this increase was associated with an increase of pollen sterility (Pagliarini, 2000). The origin of cytomixis is not clear, although the recent evidence suggest that it is genetically controlled phenomenon, which is influenced by both physiological and environmental factors (Bellucci et al., 2003). These factors could affect the occurrence of cytomixis which was observed all clones, but predominantly in the second year of study.

No matter the fact that only an exchange of cytoplasmic genetic material or relocation of chromosomes from cell to cell were observed sometimes complete genome fusion of two or three adjacent cells occurred as well. This was determined based on the pollen grain diameter, since it is well known cell size increases with increasing DNA content (Wang et al., 2010). Maybe these syncytes are formed by suppression of cell wall formation during premeiotic mitoses (so called 'archesporial syncytes'), or by fusion among microsporocytes caused by dissolution of the cell wall at the prophase I where its genomes occur coalesced, forming hyperploid nuclei (Nirmala and Rao, 1996).

The assumption is that the occurrence of collapsed microspores is mostly influenced by climatic factors. Collapsed microspores have occurred in the form of monads, triads and tetrads, although there have been cases that only one of the four microspores within callose layer was collapsed, while the other three showed normal size and appearance. Since in our study anomalies showed up in the year which was preceded by a dry autumn and irregular winter sleep, environmental factors most probably had a crucial importance. The appearance of the collapsed microspores can be caused even by cytomixis (Bhat et al., 2006).

One of the major factors that affect the level of fertility in sour cherry is the viability of the gametes, as sour cherry fruit set requires successful fertilization and at least initial growth of the zygote. *In vitro* germination tests have been used as a powerful tool for genetic, physiological, biochemical and cytochemical studies. Although in highly optimized germination media, *in vitro* tubes reach only 30–40% of *in vivo* lengths, those tests are good predictor of pollen behavior for both autotrophic phase of pollen growth where the initial steps of pollen germination and pollen tube growth utilizes internal pollen resources (Hormaza and Herrero, 1999), and a heterotrophic, rapid growth period, which uses nutrients from the transmitting tissue. No matter the fact that significant differences were found between clones II/2 and III/9 on one side and XI/3 and XIII/1 on other side, all obtained data are in the range of previously reported results for different sour cherry cultivars (Szpadzik et al., 2008; Tosun and Koyuncu, 2007). According to Dys (1984) and Pagliarini (2000) it was expectable that abnormalities in the process of meiosis (especially the deviation in chromosome number) can lead to the formation of microgametophytes with very low vitality whose direct consequence is the lower *in vitro* pollen germination. Oppositely to that, Sapre and Deshpande (1987) in some plant species proved that the absence of negative correlation between some type of anomaly, such as cytomixis and *in vitro* pollen germination, where all together leaded to quite satisfactory pollen viability. Similar results were obtained in our study where pollen germination did not correlated with none of the anomalies studied herein (data not showed). Actually, high percentage of pollen germination does not necessarily mean that all germinated pollen grains will reach the ovule and make double fertilization. We assume that the pollen grains that undergo irregular microsporogeneis, *in vivo* produce pollen tubes that are not competitive enough, and which growth will be stopped somewhere in the pistil. We expect that the real consequence of the anomalies during meiosis would manifest through the decline in fruit set, which is also proved in some other plants by Defani-Scoarize et al. (1995), Khazanehdari and Jones, (1997) and Ontivero et al. (2005).

The trait that was under the strong influence of the studied anomalies was pollen tube length. In the studied Oblacinska sour cherry clones it significantly varied. This variation could be, according to Bhat et al. (2012), a result of a meiosis regularity, activation of certain enzyme systems present in the pollen grain itself, or even carbohydrate and amino-acid concentration in pollen grain. In this study clones with high pollen germination percentage have shown higher pollen tube length too, which goes in a line with the facts concluded by Pırlak and Bolat, 1999 for apricot.

The process of meiosis in all four clones during microsporogenesis was irregular, but in varying degrees. In all studied clones the most common anomaly in Metaphase I was abnormal conjunction of chromosomes, in Telophase I and II single lagging chromosome and in tetrad-phase abnormal tetrads. Different meiosis irregularities, measured as the percentage of various anomalies during all phases and express through different frequency of microspore groups in 'tetrad' stage, were much less in clones II/2 and III/9 than the other two clones (XI/3 and XIII/1). As it was expected, characteristics of the process of meiotic stages during microsporogenesis revealed that the variations in the degree of meiotic irregularities are associated with different *in vitro* pollen germination and pollen tube length of the clones of 'Oblačinska' sour cherry. Abnormalities in the process of meiosis can lead to the formation of microgametes with low vitality, which may, therefore, reflect on the process of fertilization, especially in the case of self-fertilization.

Conflict of interest

The authors declare no competing financial interest.

Data archiving statement

We have submitted no data to public databases.

Accessions list: II/2, III/9, XI/3 and XIII/1. All accessions involved in this research are Oblacinska sour cherry clones.

Acknowledgments

This study was supported by the FP7 Project AREA316004 and Serbian Ministry of Education, Science and Technology Development Project (TR 31063). We express our sincere gratitude to Dr Amy Iezzoni from Michigan State University, USA, for encouraging

us in writing this study, fruitful discussion and critically reading the manuscript.

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