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Original scientific paper

VITALITY AND *IN VITRO* POLLEN GERMINATION OF DIFFERENT 'OBLAČINSKA' SOUR CHERRY CLONES

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Vitality of pollen, *in vitro* pollen germination and pollen tube growth (pollen tube length and pollen tube growth rate) were investigated in Oblačinska sour cherry in order to determine the differences between clones which have divergent yielding potential. For this purpose two 'Oblačinska' sour cherry clones with high fruit set and high yields (II/2, III/9) and two with low fruit set and low-yielding (XI/3 and XIII/1) were used in this study. Pollen germination was done on artificial medium containing 14% sucrose and 0.3% agar-agar at room temperature (23°C). Pollen tube growth was stopped with a drop of 40% formaldehyde, 1, 3, 6, 12 and 24 h after contact with the medium. The maximum percentage of germination ranged from 13.01% (clone II/2, after 1 h) to 54.19% (clone III/9, after 24 h). Pollen tube length varied from 64.84 μm (clone XIII/1, after 1 h) to >1,100 μm (clones II/2 and III/9, after 24 h). Pollen growth rate was quite high (up to 1.71 μm min^{-1}) after 6 h of germination, but rather decreasing until 24 h of germination (0.56–0.83 μm min^{-1}). The dynamics of *in vitro* pollen tubes growth among the clones were quite different, especially after 12 h and 24 h of germination. Clones that are singled out as fruitful (II/2 and III/9) gave much better results regarding pollen germination and pollen tube growth in comparison to clones which were characterized by low fruit set and yields (XI/3 and XIII/1).

Key words: *Prunus cerasus* L., genotype, acetocarmine, germination medium, pollen tube growth, pollen growth rate.

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INTRODUCTION

Pollen performance, which comprises pollen germination, pollen tube growth rate and pollen competition, is a prerequisite for fertilization to occur in seed producing plants, including sour cherry (*Prunus cerasus* L.). A reduction in pollen performance diminishes the fitness of the male parent through a reduction on its contribution to the next sporophytic generation. Studying and understanding the reproductive phases are crucial for agricultural practice. As in other fruit trees, pollination, stigma receptivity, pollen germination, pollen tube growth, effective pollination period and ovule fertilization are all important factors affecting both fruit set and fruit yield in sour cherry (*Prunus cerasus* L.) (SZPADZIK *et al*., 2008; FOTIRIĆ AKŠIĆ *et al*., 2013). Consequently, these aspects of reproductive biology are critical for optimizing yields in sour cherries and make them important criteria for breeding programmes (SHARAFI, 2011).

Pollen is one of the most sensitive cell systems in plants (VAN DER PLOEG and HEUVELINK, 2005), so adverse changes in the level of specific metabolites, as well as response to stresses, affect its vitality, which can lead to significant decreases in fruit production (MATSUI and OMASA, 2002; FIRON *et al*., 2006; SATO *et al*., 2006). Pollen performance criteria (pollen vitality, germination and pollen tube growth rate) are critical for discharging male gametes in the embryo sac and are a prerequisite for fertilization and fruit set (GUDADHE and DHORAN, 2012). After pollination with compatible pollen, only the fastest growing, most vigorous pollen grain will result in fertilization. Seeds which are produced after double fertilization by relatively fast-growing pollen will be more vigorous than those whose pollen donor had slow-growing pollen tube (ZHANG *et al*., 2010). Also, the concept introduced by WILLIAMS (1965), called effective pollination period (EPP), which represents the number of days during which pollination is effective in producing a fruit and determines the longevity of the ovules minus the time lag between pollination and fertilization, shows the direct correlation between pollen vitality and fruit set. However, studies on pollen are often hard because gamete development and fertilization are complex processes that occur during a short time, and are predominantly hidden within flower tissues (ZINN *et al*., 2010).

In vitro germination studies are powerful tools for genetic, physiological, biochemical and cytochemical studies for a wide range of plant species belonging to different families (RADIČEVIĆ *et al*., 2013). These studies are also a good predictor of *in vivo* pollen behavior but only for autotrophic phase of pollen growth where the initial steps of pollen germination and pollen tube growth are independent of stylar nutrients, sugars and plant growth regulators. Not only do they help with selections for breeding programmes, *in vitro* assessments can also help to predict possible problems of sterility of that particular genotype in commercial orchards. Many studies with very different experimental designs have been performed on stone fruit species, with the particular goal of investigating pollen vitality and germination (YAEGAKI *et al.*, 2002; HEDLY *et al*., 2004; WOLUKAU *et al*., 2004; DU *et al*., 2006; SHARAFI *et al*., 2010). Based on aforementioned investigations, factors known to affect *in vitro* studies include composition of the medium and ambient temperatures.

In vivo, when pollen enters a heterotrophic phase, pollen tube growth depends on numerous conditions of the transmitting tissue of the style and male-female combinations. Furthermore, regulation of microsporogenesis is positively correlated with *in vitro* pollen germination (FOTIRIĆ AKŠIĆ *et al*., 2016).

The 'Oblačinska' sour cherry (*Prunus cerasus* L.) is an autochthonous cultivar from the Balkan region. Due to long-term cultivation in diverse agro-ecological conditions and the use of

both vegetative and generative propagation, numerous genotypes are registered (RAKONJAC *et al*., 2010). Variability in tree vigour, pomological traits (fruit size, stem length, fruit quality), fruit set and yields exist among the different clones (NIKOLIĆ *et al*., 2005; MILETIĆ *et al*., 2009; ALRGEI *et al.*, 2016).

Despite the 'Oblačinska' sour cherry being self-fertile (MIŠIĆ, 2002; NIKOLIĆ *et al.*, 2005), selected genotypes showed different durations of the effective pollination period (FOTIRIĆ AKŠIĆ *et al.*, 2014). Consequently, fruit set and yields were irregular, ranging from 19 to 42% and from 4.2 to 14 kg/tree, respectively (RAKONJAC *et al*., 2010). Since Oblačinska sour cherry is self-compatible cultivar, the aim of this study was to determine *in vitro* pollen vitality, germination and pollen tube growth rate in order to determine the differences between clones which have divergent yielding potential.

MATERIALS AND METHODS

Pollen samples of different 'Oblačinska' sour cherry clones were collected in 2013, from the orchard at the Experimental Station Radmilovac, Faculty of Agriculture of the University of Belgrade, located 8 km North-East of Belgrade (44º45'N and 20º35'E, at 135 m altitude). Trees were planted in 1993. Planting distance was 4 × 2 m and trees were trained as free spindles. Trees were not irrigated. Four genotypes were selected based on different fruit set and yield data, during 2006–2008 (RAKONJAC *et al.* 2010). Namely, the genotypes II/2 and III/9 resulted in markedly high fruit set and large yields, while genotypes XI/3 and XIII/1 had low fruit set and yields.

Flower clusters at the balloon stage (stage 59 according to the BBCH scale, MEIER *et al*., 1994) were collected from all four 'Oblačinska' sour cherry clones, transported to the laboratory and placed in jars with water and kept at room temperature (23 ± 2°C). Anthers were collected just before dehiscence. Undehisced anthers were dried at room temperature for 24 h to induce dehiscence.

Pollen vitality was tested in 1% acetocarmine solution. Under the Leica DM LS microscope (Leica Microsystems, Wetzlar, Federal Republic of Germany), orange stained and normal looking pollen grains were considered to be viable whereas, shriveled, slightly stained or colorless pollen grains were counted as non-viable. Three microscopic repetitions of each clone were observed and number of viable and non-viable pollen grains was counted in each field and vitality percentages were calculated.

In vitro germination percentages of pollen were obtained using Petri-dishes with medium containing agar (0.3%) and sucrose (14%). Pollen grains were dispensed onto the germination medium with a brush. Petri dishes were incubated at room temperature, and after 1, 3, 6, 12 and 24 h pollen tube growth was stopped with a drop of 40% formaldehyde. Percentage germination of pollen grains (n=200) were evaluated under a Leica DM LS microscope where pollen grains were considered as germinated if pollen tube length was greater than the diameter of the pollen grain. Pollen tube length was measured for all four genotypes and in all treatments, using the 'Leica IM 100' program. For every replicate, length of 30 pollen tubes were measured. Pollen growth rate (*PGR*) was calculated using the following equations:

$$PGR = \frac{L}{t}$$

where L is the pollen tube length; t is a time of incubation.

Data were analyzed by ANOVA and significance of differences among the assessed sour cherry clones was done with LSD test using the software program Statistica 5.0 (StatSoft, Inc., Tulsa, OK, USA).

RESULTS AND DISCUSSION

Staining of pollen grains with 1% acetocarmine found significant differences between clones (Figure 1). Clones II/2 and III/9 had the pollen vitality at the same level (80.6%) and significantly higher vitality in comparison to the clones XI/3 and XIII/1 (54.9% and 52.9%, respectively). These results were consistent with those of SZPADZIK *et al.* (2010), who determined pollen vitality of Hungarian sour cherry cultivars in Polish agro-climatic conditions and obtained the range from 55% ('Újfehértói Fürtös') to 84% ('Koral'). Although staining is not always a reliable test for pollen but, in combination with *in vitro* germination tests they can provide a better understanding of pollen performance (BHAT *et al.*, 2012).

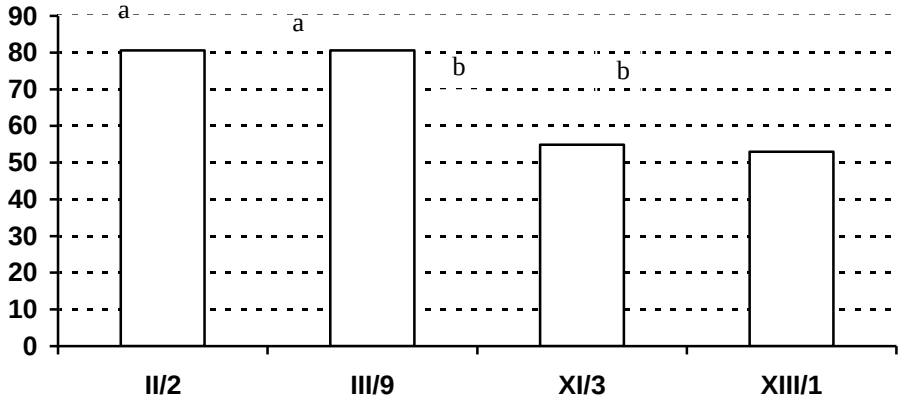

Figure 1. Pollen vitality (%) in four 'Oblačinska' sour cherry clones
*Significant differences are marked with different letters at $p<0.05$

Average pollen germination of all four 'Oblačinska' sour cherry clones increased as incubation time was prolonged (Table 1). Initially, the assessed clones had the same level of germination, but as the time passed, the average pollen germination elevated from 15.45% (1 h of incubation) to 45.24% (24 h of incubation). Statistically significant differences appeared after 12 h of incubation, where greater numbers of pollen of clones II/2 and III/9 (Figure 2a) (42.08% and 42.49%, respectively) germinated compared to pollen from clones XI/3 and XIII/1 (32.33% and 33.40%, respectively) (Figure 2b). Indeed, after 24 h the trend continued for pollen originated from clones II/2 and III/9 (48.76% and 54.19%, respectively). The range for percentage of pollen germination reported in this work was in agreement with the findings of TOSUN and KOYUNCU (2007) and SZPADZIK *et al.* (2008). Differences in pollen germination might be due to differences in the nutritive status of the developing pollen grains, affected by environmental conditions (extremely high and/or excessive cold) and even inbreeding in the

previous sporophytic generation (BEYHAN and KARAKAŞ, 2009; HASANUZZAMAN *et al*., 2013; PAUPIÈRE *et al*., 2014).

Table 1. Pollen germination (%) ± SE (standard error) in four 'Oblačinska' sour cherry clones at different incubation times.

Clone	Incubation time				
	1 hours	3 hours	6 hours	12 hours	24 hours
II/2	13.01±1.7	19.93±3.4	32.29±3.4	42.08±2.5b*	48.76±2.1c
III/9	18.77±1.9	22.71±2.8	29.74±1.6	42.49±3.1b	54.19±1.0d
XI/3	14.83±0.4	21.38±0.2	31.01±2.3	32.33±2.3a	36.96±3.6a
XIII/1	15.20±1.6	27.15±3.3	32.50±4.2	33.40±0.2a	41.04±1.5b
Average	15.45±1.4	22.79±2.4	31.39±2.9	37.58±2.0	45.24±2.0

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

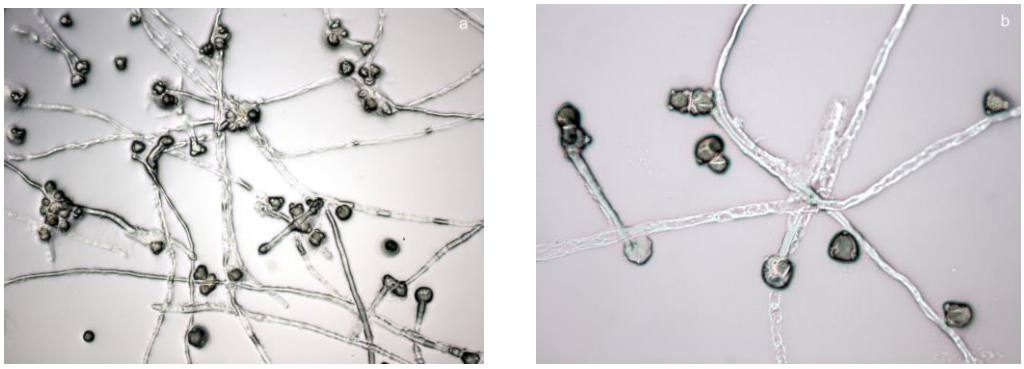

Figure 2. *In vitro* pollen germination in 'Oblačinska' sour cherry clones II/2 after 24 h of incubation (a) and XI/3 24 h of incubation (b).

Similar to pollen germination, the average length of pollen tubes (Table 2) increased as the incubation period progressed, and ranged from 79.79 μm (1 h of incubation) to 994.38 μm (24 h of incubation). The growth rate in all clones was almost identical in the first 12 h after incubation, continued at the same rate for clones II/2 and III/9, but slowed down significantly for clones XI/3 and XIII/1. Statistical analyses however proved only significant differences between pollen tube growth lengths after 24 h of incubation. The shortest pollen tube length was 64.84 μm (clone XIII/1, after 1 h of incubation) and the longest being 1,189.24 μm (clone II/2, after 24 h of incubation). These results were in agreement with results reported by SHARAFI (2011), where pollen tube length in sweet cherry and sour cherry were between 327 μm to 956.1 μm, and between 217.1 μm up to 623.2 μm, respectively. On the other hand, HUI-JUAN *et al*. (2008) detected much greater pollen tube length in *Prunus salicina* (up to 1.1 mm after 6 h of incubation). These distinctions among the different species could be a result of a

microsporogenesis regularity (FOTIRIĆ AKŠIĆ *et al.*, 2016), activation of certain enzyme systems present in the pollen grain itself (BHAT *et al.*, 2012), or even carbohydrate and amino-acid concentration in it (CASTRO and CLÉMENT, 2007; MATTIOLI *et al.*, 2012).

Table 2. In vitro average pollen tube length (μm) ± SE (standard error) in four 'Oblačinska' sour cherry clones at different incubation times.

Clone	Incubation time				
	1 hours	3 hours	6 hours	12 hours	24 hours
II/2	81.05±6.8	274.52±16.8	616.13±15.5	685.09±39.6	1,189.24±48.8b*
III/9	91.50±4.9	290.84±13.9	602.89±14.1	839.11±59.1	1,126.30±55.7b
XI/3	81.78±2.9	252.84±10.7	535.82±14.6	756.27±33.1	862.10±58.1a
XIII/1	64.84±4.0	270.68±8.2	435.49±21.8	767.94±20.0	799.90±20.3a
Average	79,79±1.6	272,22±12.4	547,67±16.5	762,10±37.9	994.38±45,7

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

'Oblačinska' sour cherry clones with poor fruit set and low yields, described in previous studies, had lower rates of pollen germination, while genotypes with both high fruit set and yields had higher rates of pollen germination. Although staining tests resulted in higher values than the percentage germination, were positively correlated with pollen tube growth of clones II/2 and III/9 being significantly greater than that pollen from clones IX/3 and XIII/1 which confirms that staining is suitable predictor for how pollen grains will behave during germination.

Table 3. In vitro pollen tube growth rate (μm/min) ± SE (standard error) of four 'Oblačinska' sour cherry clones at different incubation times.

Clone	Incubation time					Avegare
	1 hours	3 hours	6 hours	12 hours	24 hours	
II/2	1.35±0.1b*	1.53±0.2	1.71±0.7	0.95±0.2	0.83±0.2d	1.27±0.3c
III/9	1.53±0.3c	1.62±0.5	1.67±0.4	1.17±0.2	0.78±0.0c	1.35±0.3d
XI/3	1.36±0.0b	1.40±0.6	1.49±0.0	1.05±0.3	0.60±0.1b	1.18±0.2b
XIII/1	1.08±0.2a	1.50±0.4	1.21±0.4	1.07±0.2	0.56±0.2a	1.08±0.3a
Average	1.33±0.2	1.51±0.4	1.52±0.4	1.06±0.2	0.62±0.1	1.21±0.3

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

Since the initial steps of pollen germination occur in an autotrophic way at the expense of the pollen grain reserves and it is independent of stylar nutrients (HERRERO, 2000), the pollen tube growth *in vivo*, in some pollination combinations in certain fruit crops, directly affects the degree of fertilization (CEROVIĆ and MIĆIĆ, 1999). The rate at which pollen tubes elongate differs widely between species and also between *in vivo* and *in vitro* conditions (STONE *et al.*, 2004). The evaluation of pollen tube growth using sequential incubation times confirmed that all examined 'Oblačinska' sour cherry clones had similar growth rates (Table 3). The rate of pollen tube growth increased from 1 h to 6 h of incubation, where the average pollen growth rate in all

four 'Oblačinska' sour cherry clones was 1.33 μm min^{-1} and 1.52 μm min^{-1}, respectively. Subsequently, after 12 h and 24 h of incubation pollen tube growth rates decreased, probably due to depletion of nutrients within the pollen grains. After 24 h of incubation, averagely clone III/9 exhibited the highest pollen tube growth rate (1.35 μm min^{-1}), whereas clone XIII/1 had the lowest rate (1.08 μm min^{-1}). Analysis of variance showed that during 1 h and 24 h of incubation, clones presented significantly different pollen tube growth rate.

CONCLUSION

In this study, the examination of *in vitro* pollen germination of the selected 'Oblačinska' sour cherry clones suggested that pollen performance is strongly influenced by genotype. The clones with both high fruit set and large yields, had higher pollen vitality (around 80%), pollen germination (48.76–54.19%), pollen tube length (>1,100 μm) and pollen tube growth rate (1.27–1.35 μm min^{-1}). On the contrary, 'Oblačinska' sour cherry clones which were characterized by poor fruit set and low yields had much lower pollen performance traits. It can be concluded that *in vitro* pollen germination and performance are positively correlated with yield productivity in commercial orchards. Therefore, our results suggest that the genotypes with high pollen performance could be grown in single-cultivar orchards, and may act as efficient pollenizers for other high quality cultivars assuming there are no incompatible alleles.

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VITALNOST POLENA I *IN VITRO* PORAST POLENOVE CEVČICE KOD NEKIH KLONOVA OBLAČINSKE VIŠNJE

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Izvod

Osobine polena koje obuhvataju klijavost, porast i međusobnu kompeticiju je preduslov za obrazovanje semena kod biljaka. Smanjeni potencijal polenovg zrna umanjuje šansu prenošenja gena muškog roditelja u sledećoj sporofit generaciju. U ovom radu ispitivani su vitalnost polena, *in vitro* klijavost polena i porast polenove cevčice (dužina polenove cevčice i brzina rasta) kod četiri klona Oblačinske višnje (II/2, III/9, XI/3 i XIII/1). Veštačka podloga za ispitivanje klijavosti polena sadržala je 14% saharoze i 0,3% agar-agara. Porast polenove cevčice zaustavljen je 1, 3, 6, 12 i 24 časa nakon kontakta polena sa medijumom dodavanjem jedne kapi 40% formaldehida. Maksimalna klijavost polena kretala se od 13.01% (kod klona II/2, posle 1 h inkubacije) do 54.19% (klon III/9, posle 24 h). Dužina polenove cevčice varirala je od 64.84 μm kod klona XIII/1, posle 1 h, do >1100 μm kod klonova II/2 i III/9 posle 24 h. Brzina porasta polenove cevčice bila je velika (do 1.71 μm min^{-1}) posle 3 h naklijavanja, ali je naglo opadala do 24 h naklijavanja (0.56-0.83 μm min^{-1}). Brzina *in vitro* porasta polenove cevčice između klonova bila je značajno različita, posebno između 24 i 48 h inkubacije na hranljivoj podlozi, što je utvrđeno analizom varijanse. Klonovi koji su bili izdvojeni kao rodni (II/2 i III/9) pokazali su mnogo bolje rezultate klijavosti polena i porasta polenove cevčice u odnosu na klonove koji su davali slabije prinose (XI/3 i XIII/1).

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