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In vitro performance of fresh and stored plum (*Prunus domestica* L.) pollen

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Abstract. In this study, the *in vitro* performance of fresh and pollen stored for three months at different constant temperatures was investigated in three plum cultivars ('Valerija', 'Čačanska Lepotica', 'Valjevka'). Pollen was collected from flowers in the late balloon phase and, after drying, stored for three months at four different constant temperatures (+4°C, -20°C, -80°C, -196°C). Pollen tube length as well as the nuclear presence in the pollen tube and its migration were examined. *In vitro* pollen tube length was tested after a 24-hour germination period, after which the pollen tube was stained with Hoechst 33258 to observe the nuclear presence within it. The longest average pollen tube length in all tested cultivars was observed with fresh pollen. With the pollen stored at low temperatures, a longer pollen tube was obtained compared to pollen stored at +4°C. In all cultivars, mature pollen grains at the binucleate stage contained a vegetative nucleus (VN) and a generative nucleus (GN). Although at each storage temperature a higher percentage of GN was achieved in pollen tubes compared to fresh ones, nevertheless, regular movement of nuclei, primarily VN rather than GN or sperm nucleus (SN), was found in the highest percentage in all tested cultivars.

Key words: European plum, pollen tube, vegetative nucleus, generative nucleus, sperm nucleus

Introduction

The fruits of common European plum occupy a significant place in the market for fresh products as well as in various types of processing. In this regard, different breeding objectives have been established in plum breeding centers in Europe to meet the needs of producers, markets, and consumers (Butac, 2020). In a breeding programme determining the viability and germination potential of pollen has great importance. In such programs, the need to store pollen without losing germinability and fertility arises when it comes to larger hybridizations, in the case of nonsynchronous flowering and/or geographical isolation of parental species, or when dealing with the low vitality of currently available fresh pollen. So far, there are several reports in different fruit species showing that pollen stored at low temperatures is effective for short- or long-term preservation period (Martínez-Gómez et al., 2002; Alburquerque et al., 2007; Beltrán et al., 2019; Čalić et al., 2021; Đorđević et al., 2022).

About 70% of flowering plants disperse pollen at the binucleate stage, consisting of a vegetative nucleus and a generative nucleus – the latter being the precursor of the two sperm cells (Brewbaker, 1967). In contrast to trinucleate pollen grain, binucleate is less differentiated, longer-lived, and latently inefficient in pollination environments (Williams et al., 2014). During pollen tube elongation, the vegetative nucleus and the sperm cells are usually closely associated and move as a 'male germ unit' (MGU). There is little data available on the presence of nuclei in pollen grains after storage perod (Čalić et al., 2021), and there has been no previous report on their presence and distribution in the pollen tube after germination. Thus, the main objective of this work is to analyse the length of the pollen tube and the presence of nucleus in it after a short storage period at different constant temperatures in three plum cultivars.

Materials and Methods

Plant material. Three plum cultivars with different flowering periods, 'Valerija', 'Čačanska Lepotica' and 'Valjevka', were used in the present study. During the spring of 2017, anthers were collected from the flower buds immediately before anthesis. Anthers were collected in paper bags and dried for several days at room temperature. After drying, anthers were put into marked vials and left for three months in the refrigerator (+4°C), freezer (-20°C, -80°C), and liguid N (-196°C). After the storage period, pre-hydration was performed for pollen stored at low (incubation at room temperature for a short period) and ultra-low (fast thawing) constant temperatures.

Pollen tube length. The determination of the pollen tube length of fresh pollen as well as those stored for three months at constant temperatures was based on the observation of germinated pollen tubes on *in vitro* nutrition medium. The length of the pollen tube was observed after a 24-ourh incubation period at 25°C. The furder growth of the pollen tube after the incubation period was stopped with a drop of formaldexide. Measurements were made using the Analysis software programme (Olympus, Tokyo, Japan) under a light microscope. For fresh pollen and pollen stored at different temperatures the length was measured on 30 randomly selected pollen tubes.

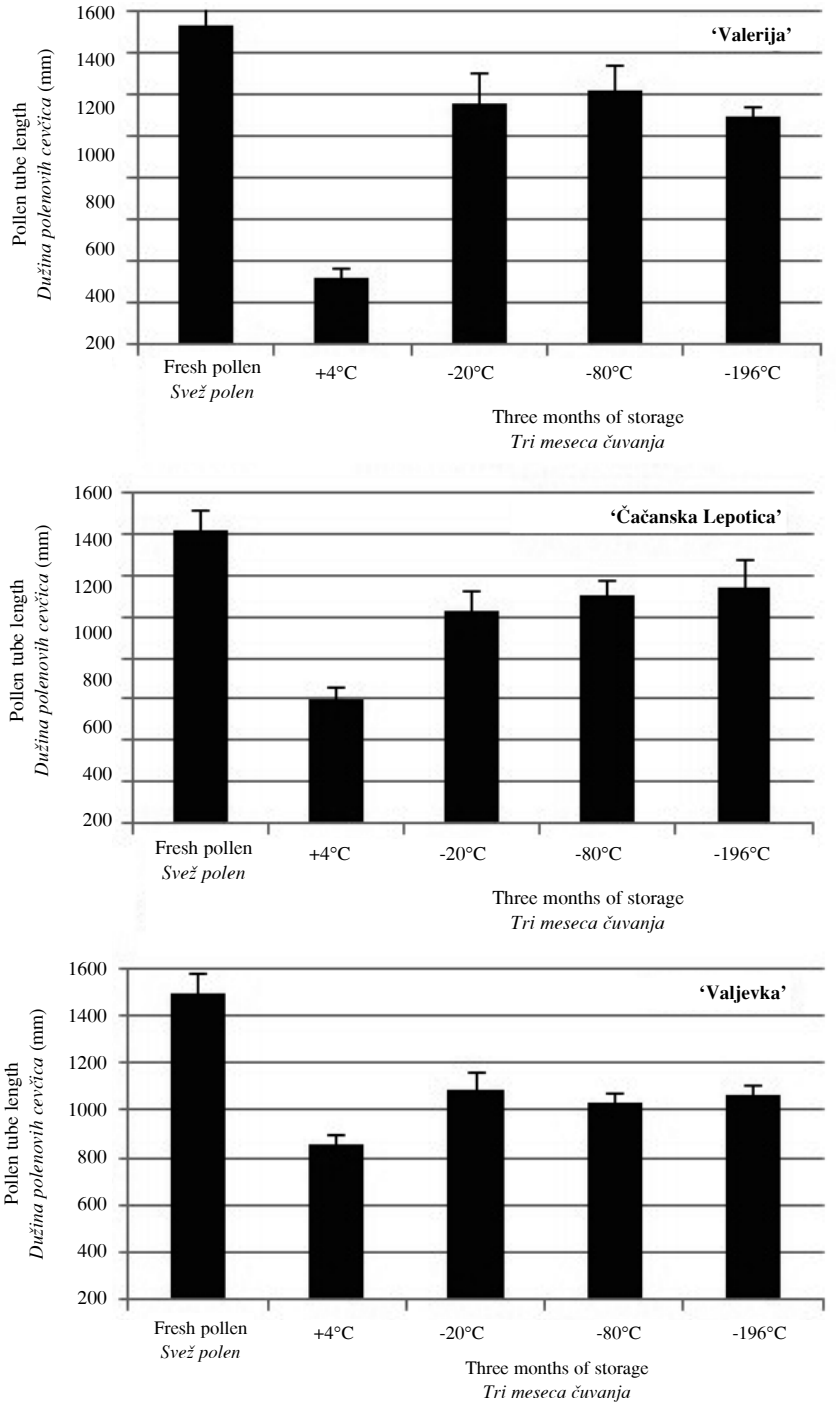
Staining procedure. To assess nuclear events in pollen grains and pollen tubes, fluorescent staining with Hoechst 33258 (H-33258) was carried out according to the method described by Hough et al. (1985). After staining, the distribution of vegetative nuclei (VN), generative nuclei (GN), and sperm nuclei (SN) was observed under UV light (blue fluorescence) with an Olympus BX61 fluorescent microscope (Tokyo, Japan). The distribution of nuclei in the pollen tube was tested in an average of 30 pollen tubes.

Statistical analysis. Statistical analysis of the results was performed using STATISTICA for Windows 8.0 (StatSoft Inc., Tulsa, Okland, USA).

Results and Discussion

Factors such as the physiological condition of the tree as well as environmental conditions can cause variation in pollen germination and pollen tube length (Impre et al., 2020). Also, the composition of the medium has a great influence on pollen germination and pollen tube length (Peng et al., 2015). Pollen tube elongation represents the ability of pollen to germinate, so pollen tube lengths *in vitro* were measured. The mean pollen tube length of fresh pollen grains after a 24-hour incubation period among the studied plum cultivars was 1297.99–1523.39 µm. The longest mean pollen tube length of fresh pollen was obtained in 'Valerija' (Graph 1), while the lowest was in 'Valjevka'. Previous results in plum indicate wide variability in relation to the mean pollen tube length of mature pollen (Sharafi, 2011; Sharafi et al., 2013; Milatović et al., 2015; Beltrán et al., 2019).

Unfreezing and rehydration of pollen stored at ultra-low temperatures are very important steps to observe better pollen germination rates *in vitro* (Peng et al., 2015). After a three-month storage period at different constant temperatures, the mean pollen tube length in all cultivars was lower than the values obtained for fresh pollen grains. The rapidly decreasing length of the pollen tube was observed with pollen stored at +4°C (< 670 µm). In this study, the mean pollen tube length after storage at low and ultra-low temperatures (-20°C, -80°C and -196°C) in each cultivars was approximately the same. In 'Valerija' and 'Čačanska Lepotica' the length of the pollen tube was more than 1000 µm at all constant temperatures, while in 'Val-



Graph 1. Pollen tube length of fresh pollen and pollen stored at different constant temperatures. Bars present means + SE, n = 30
Grafik 1. Dužina polenovih cevčica svežeg polena i polena čuvanog na različitim konstantnim temperaturama. Trake u grafikonima označavaju prosek + SE, n = 30

jevka' more than 800 µm. As a result of this, low and ultra-low temperatures were found to be suitable for short periods of pollen storage since pollen grains maintain high germinability. A previous study about the effect of temperature storage conditions on pollen *in vitro* performance in apple, cherry, pear, and plum in-

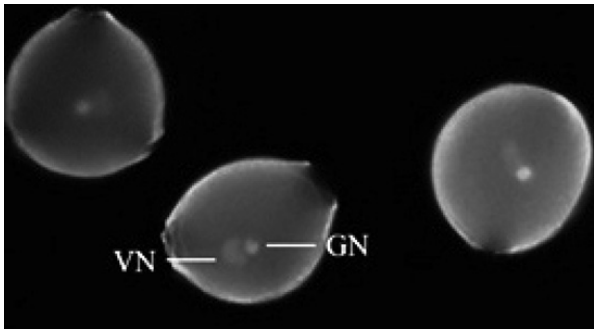


Figure 1. Binuclear pollen grains of plum cultivar 'Valerija' (VN – vegetative nucelus; GN - generative nucelus)
Slika 1. Dvojedarna polenova zrna sorte šljive Valerija (VN – vegetativno jedro; GN - generativno jedro)

dicated that the greatest mean and maximum pollen tube length were obtained after storage for two months at -20°C (Beltrán et al., 2019).

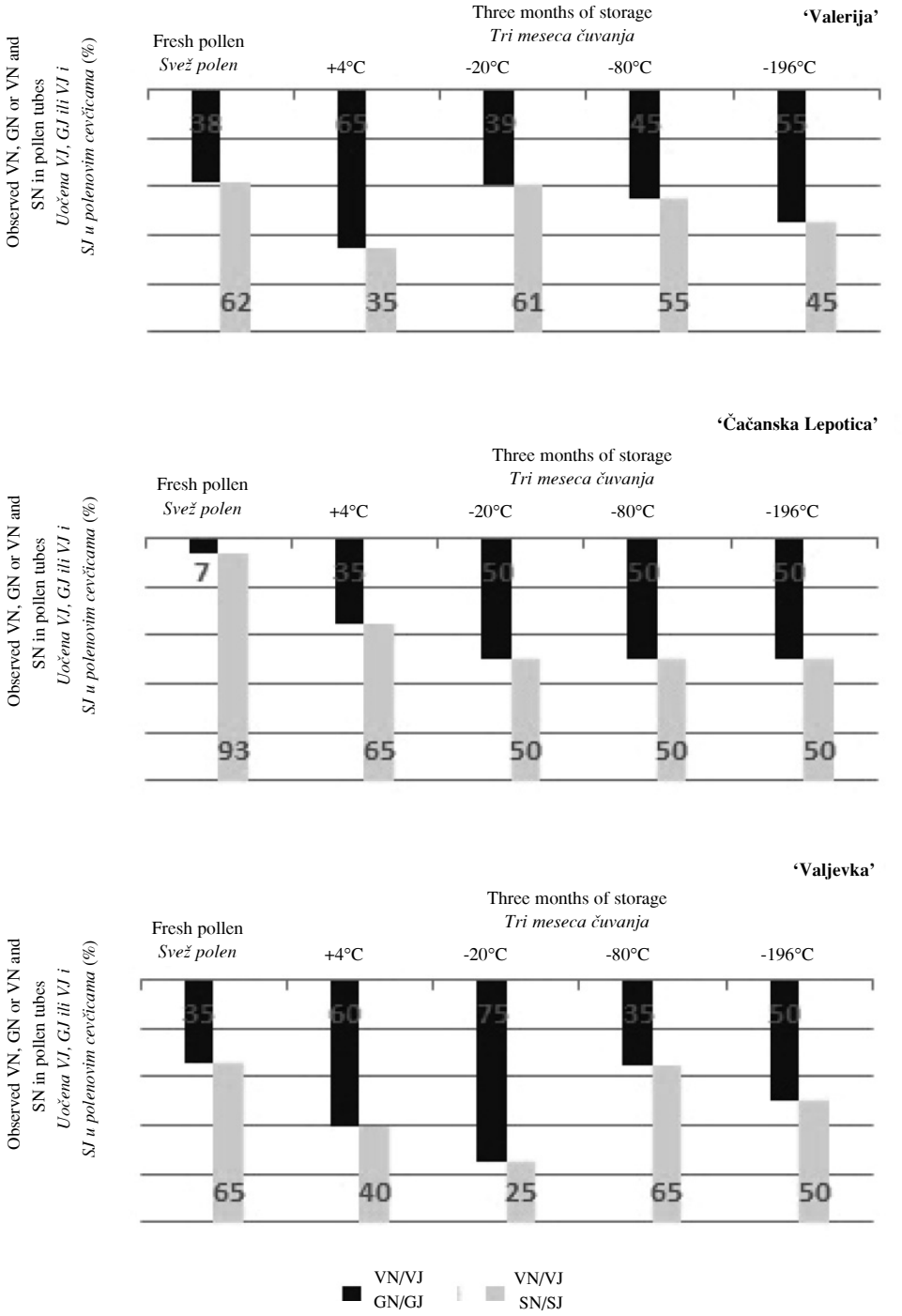
For testing nuclear status in pollen grains and in the pollen tube, the fluorescent stain Hoechst 33258 was used. No significant amount of dead pollen was observed using this staining. Analysis of nuclear number in mature pollen grains of all cultivars showed the presence of GN and VN, which are closely associated in the center of the grain (Figure 1). GN was heavily stained and much smaller than VN, which was slightly stained.

According to these findings, at anthesis, tested plum cultivars spill their pollen in a binucleate stage. The results obtained in the present study are in agreement with results obtained on plum and apple trees (Čalić et al., 2013, 2021).

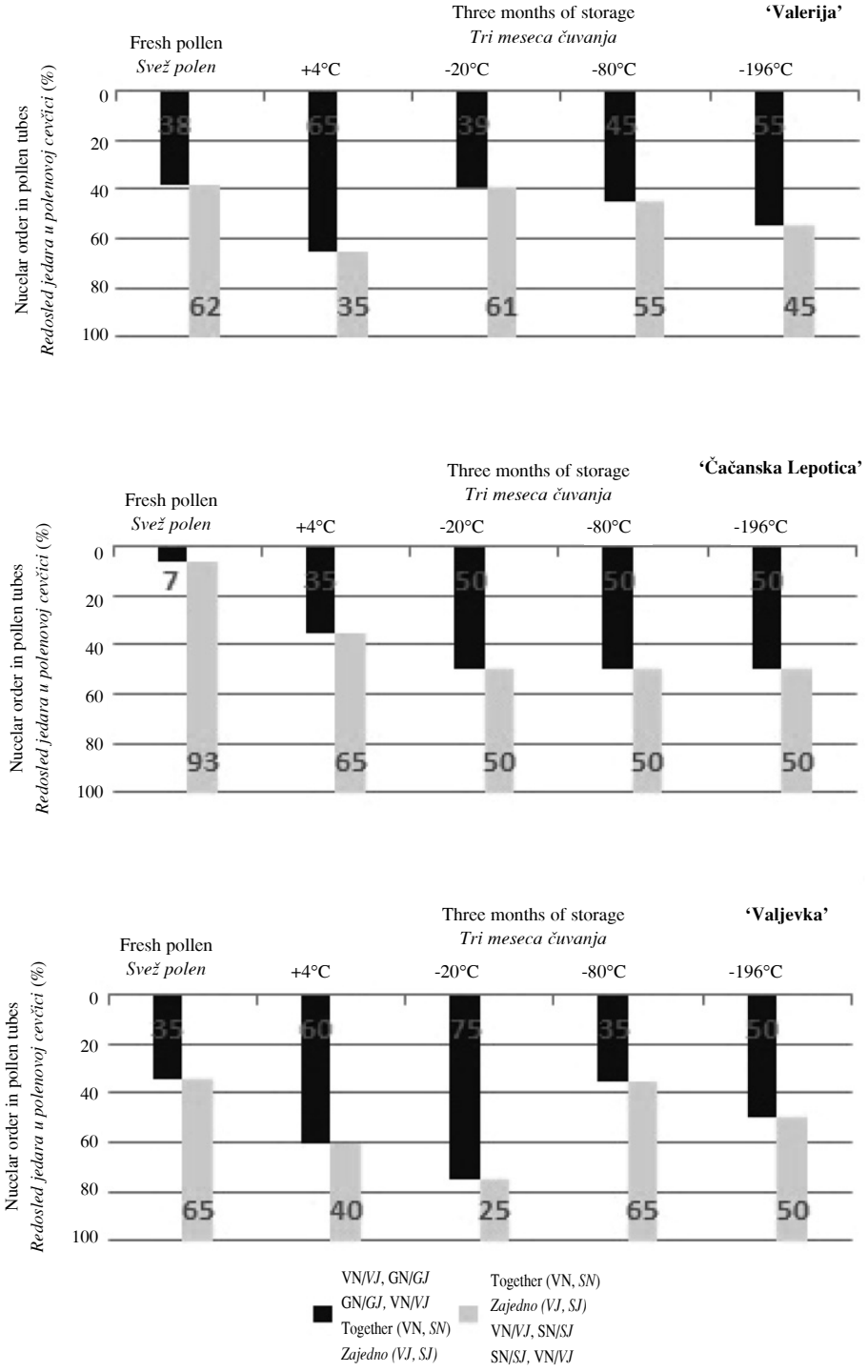
In the pollen tube, GN and SN were round and brightly fluorescent, while VN showed less intense fluorescence and was elongated, round, or irregular in shape. In all tested cultivars, after staining in the pollen tube of fresh pollen, a higher percentage of the presence of VN and SN was noticed (>60%) (Graph 2). These findings indicate that a second mitotic division takes place in the growing pollen tube. In the majority of angiosperms, division of the genetative nucleus occurs in the pollen tube following germination (Shivanna et al., 1988).

Almost all pollen tubes contained GN/SN, and one VN migrating from the grain towards the growing tip. In pollen tubes from pollen stored at different constant temperatures, a higher presence of GN was observed, indicating that mitosis of GN in stored pollen was significantly delayed relative to mitosis of GN in fresh ones. Compared to fresh pollen in the pollen tube from pollen stored at + 4°C in all cultivars, around 70% more GN was observed. For pollen stored at different constant temperatures, approximately the same values of the nuclear presence in the pollen tube were determined, except for 'Valjevka' at -20°C, where in 70% of pollen tubes GN was observed. In the pollen tube, the timing of GN mitosis has been related to some aspects of pollen tube biology, including the shift from autotrophy to heterotrophy and (possibly) self-incompatibility (Read et al., 1993). Delayed division of GN may be a result of delayed pollen germination because GN mitosis has been correlated with the duration of pollen tube growth (Brewbaker & Majumder, 1961). According to Williams et al. (1999), delayed pollen germination and slow pollen tube growth indicate poor competitive ability but not loss of fertilization potential.

We also examined the nuclear position from the tip of the pollen tube and found different arrangements of VN and GN/SN (Graph 3). In the pollen tube of fresh pollen, in most cases, the VN precedes or is joined together with the GN/SN (Figure 2A, B, D, E), which is a regular nuclear movement observed in many fruit species (Cerović, 1994; McCue et al., 2011). In all pollen tube cases where the SN was observed, two nuclei were close to each other. According to McCue et al. (2011), SN remain linked until just prior to fertilization. In very few cases, GN or two SC ahead of the VN were observed in the pollen tube of 'Valjevka' (Figure 2C, F).



Graph 2. Presence of vegetative (VN) and generative (GN) or vegetative and spermatic (SN) nuclei in pollen tubes
Grafik 2. Prisustvo vegetativnog (VJ) i generativnog (GJ) ili vegetativnog i spermatičnog (SJ) jedra u polenovim cevčicama



Graph 3. Position of the vegetative (VN), generative (GN) and sperm (SN) nuclei from the top of pollen tube
Grafik 3. Pozicija vegetativnog (VJ), generativnog (GJ) i spermatičnih (SJ) jedara od vrha polenove cevčice

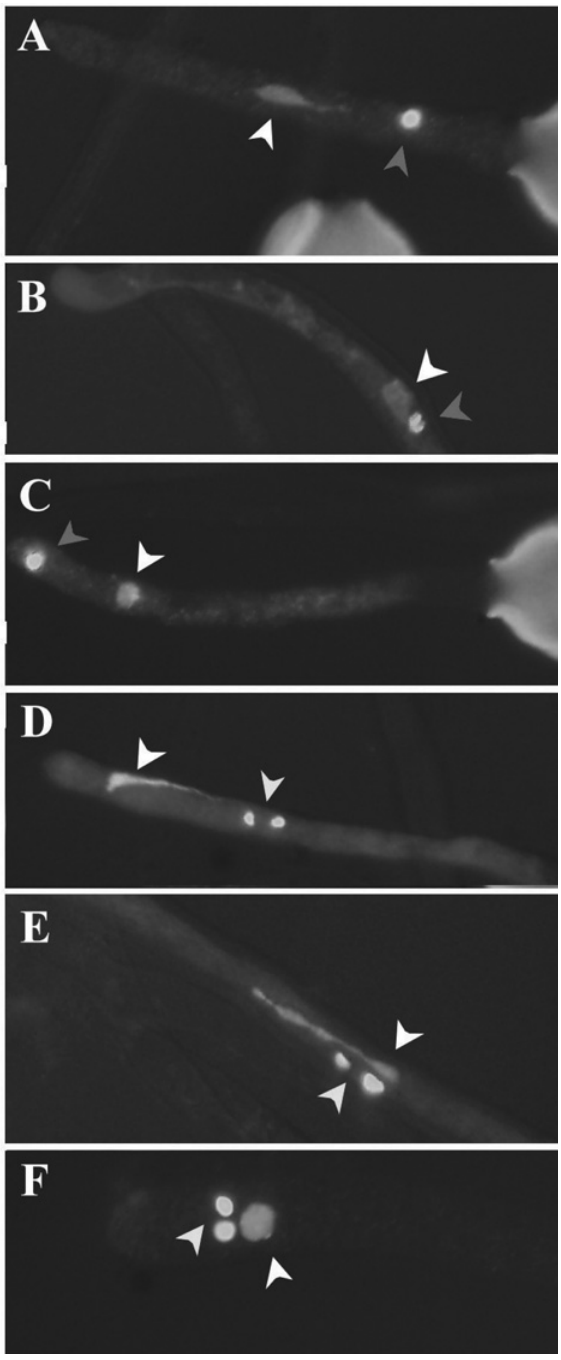


Figure 2. Nuclear order from the top of the pollen tube after 24-hour germination period: white arrows (vegetative nuclei – VN), red arrows (generative nuclei – GN), and yellow arrows (sperm nuclei – SN)
Slika 2. Raspored jedara u polenovoj cevčici nakon 24 h perioda klijanja: bela stelica (vegetativna jedra – VJ), crvena stelica (generativna jedra – GJ), žuta strelica (spermatična jedra – SJ)

A similar situation was observed in the pollen tubes from the stored pollen at different constant temperatures. In around 90% of the pollen tubes, nuclear arrangements of either VN, GN, or VN SN were found in each cultivar. Also, in cultivars, there was a noticeable reversed nuclear order (< 10%). Even when the SN were ahead and disconnected with the VN, results so far showed that the SN still moved forward, indicating an existing driving force facilitating the movement of the SN (Zhou & Meier, 2014). In a few cases, in pollen tubes of fresh and stored pollen at different constant temperatures, isolated VN and solely migrating SN were observed.

Conclusion

The results obtained herein confirmed the possibility of plum pollen storage at different constant temperatures for a short period of time without losing its vitality. The pollen of the studied plum cultivars maintains its capacity to germinate after storage, but shorter pollen tubes have been observed. The mature pollen grains of the plum cultivars are in the binucleate stage at anthesis. After the storage period, a higher percentage of GN in the pollen tube was observed in all cutivars. However, with regards to nuclear distribution in the pollen tube of fresh and stored pollen at different constant temperatures, the highest percentage of the typical order of VN first, then of GN or SN, was observed.

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In vitro PERFORMANSE SVEŽEG I ČUVANOG POLENA ŠLJIVE (*Prunus domestica* L.)

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Rezime

U ovom radu su ispitane performanse *in vitro* svežeg i polena čuvanog na različitim konstantnim temperaturana tokom tri meseca kod tri sorte šljive (Valerija, Čačanska Lepotica, Valjevka). Polen je sakupljan iz cvetova koji su bili u kasnoj balon fazi i nakon sušenja čuvan na četiri konstantne temperature (+4°C, -20°C, -80°C, -196°C). Ispitana je dužina polenovih cevčica, prisustvo i migracija jedara u njima. Dužina polenovih cevčica je određivana nakon inkubacionog perioda od 24 h, a zatim bojenje sa Hoechst 33258 da bi se uočilo prisustvo jedara u njima. Prosečno najduže polenove cevčice kod svih ispitivanih sorti utvrđene su kod svežeg polena. Kod polena čuvanog na niskim temperaturama prosečna dužina polenovih cevčica je bila veća nego kod polena čuvanog na +4°C. Polen svih sorti u zrelom stanju je dvojedaran, sadrži vegetativno i generativno jedro. Iako je kod svih sorti nakon perioda čuvanja polena, uočen veći procenat prisustva generativnog jedra u polenovim cevčicama, raspored jedara je bio regularan, prvo vegetativno, potom generativno ili spermatično jedro.

Ključne reči: Evropska šljiva, polenova cevčica, vegetativno jedro, generativno jedro, spermatično jedro