In vivo Performance of Cryotreated Pollen of Sweet Cherry (*Prunus avium* L.)

Running title: Sweet Cherry Pollen Performance after Cryotreatment

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ABSTRACT

The primary objective of the research was to reveal the effect of cryotreatment on pollen reproductive ability *in vivo* by monitoring pollen tube growth in sweet cherry (*Prunus avium* L.) pistils. The influence of cryotreatment was considered from the context of its interaction with polliniser genotype, and the possibility for usage of *in vivo* parameters for assessment of pollen fertility after it. Pistils of sweet cherry cultivar 'Regina' were pollinated with both cryotreated and fresh pollen of 'Kordia' and 'Summit'. Quantitative parameters of pollen tube growth *in vivo* (pollen tube number in certain pistil parts, pollen tube dynamics and fertilization percentage) were determined using the fluorescence microscopy method, and obtained data were analysed together with those obtained for *in vitro* pollen performance and fruit set. The research has indicated that: i) the reproductive ability of cryotreated sweet cherry pollen can be estimated by analysing parameters of its performance *in vivo*, which should be combined with *in vitro* test; ii) semi- or fully-compatible female recipients, whose sporophytes are supportive for male gametophytes should be used for the assessment; iii) the key parameters for *in vivo* testing are pollen tube number in the upper part of the style and in the ovary, as well as fertilisation percentage, i.e. the percentage of pistils with penetration of the longest pollen tube in the nucellus of the ovary on the 10th day after pollination. Therefore, *in vivo* testing of the pollen reproductive ability after cryotreatment can give answers about pollen quality with valuable credibility, in a maximum of two weeks after thawing and rehydration.

Keywords: *Prunus avium* L., Pollen, Cryotreatment, Fluorescence microscopy, Pollen tube growth.

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INTRODUCTION

Pollen of different continental fruit species is a useful source of genetic diversity and may be an important part of genebanks. Breeders often have to overcome geographical distances, which result in asynchrony in flowering time among crossing parents, by storing and sending pollen. Consequently, breeding programmes are tightly linked to pollen conservation and identification of parameters related to its fertility (Giovannini *et al.*, 2017).

Pollen is characterized by a short life span at ambient temperature which varies by species, ranging from a few hours to several months (Rajasekharan *et al.*, 2013) – depending on whether it is harsher conditions in nature, room temperature, or storage in the refrigerator. Environmental factors affect the success of pollen storage and their lowering usually leads to pollen metabolism decrease, which allows greater longevity and viability independent of the storage period (Dinato *et al.*, 2020). Long-term storage of fruit species pollen makes cross-breeding possible between the cultivars differing in flowering time, or between plants growing in remote places, resulting in extended utilization of germplasm.

Cryopreservation is a simple and highly efficient method used to conserve plant genepool components – seeds, tissue material, etc. (Engelmann, 2004; Ružić *et al.*, 2014; Vujović *et al.*, 2015), as well as pollen grains (Rajasekharan *et al.*, 2013; Dinato *et al.*, 2020). The mature male gametophyte of most plant species is desiccation tolerant, which renders them ideally suitable for storage, especially under cryogenic conditions (Rajasekharan *et al.*, 2013). Moisture content (MC) plays a major role during cryopreservation – excess moisture in pollen forms ice crystals damaging pollen membranes and causing alterations in the structural integrity of cells, resulting in mechanical and physical injury that ultimately leads to viability loss (Xu *et al.*, 2014). Unlike multicellular plant structures in which extracellular ice formation reduces the possibility of the cytoplasm freezing, pollen grains are single entities and this mechanism of protection is likely limited (Dinato *et al.*, 2020). Thus, the hydration level must be decreased before cooling and according to Connor and Towill (1993) the MC of the pollen intended for successful long-term conservation at -80°C to -196°C should be between 7% and 20%.

In recent decades, ultra-low temperatures have been used for pollen storage, particularly in liquid nitrogen (LN), either in the liquid phase (−196°C) or in the vapour phase (−150 to −180°C), retaining the original viability of pollen grains, and offering their long-term storage (Rajasekharan and Rohini, 2023). At the cryogenic temperature (-196ºC), all metabolic activities are kept under ‘suspended animation’ resulting in long-term conservation

(Engelmann, 2004). Pollen dehydrated to an optimal MC and frozen at LN temperatures has been documented to store well for over 15 years without loss of its essential capabilities to pollinate, fertilize and set fruit (Sparks and Yates, 2002; Panella *et al.*, 2009).

No standardized procedures or viability-testing protocols have been established for using cryopreserved pollen for breeding and other purposes in heterozygous and polyploid fruit tree species. Up to now, many pollen viability and germinability tests *in vitro* have been used (Martínez-Gómez *et al.*, 2002; Duta *et al.*, 2013; Čalić *et al.*, 2021), with changeable conditions of testing (pollen rehydration, staining, medium content, etc). More recent results, obtained in plums (Đorđević *et al.*, 2022), deal with the pollen performance *in vivo* after a certain period of storage. These investigations focused on pollen storage longevity itself, giving us valuable data related to the possibility of after-storage usage, but without the possibility to compare pollen performance, particularly *in vivo*, of cryotreated and fresh pollen from the same season; the only possible is comparing of one-year-stored pollen with the fresh pollen from next season, due to biological cycle. In that way, the influences of microsporogenesis and seasonal impacts that are evident on pollen *in vivo* performance (Cerović, 1991), are not excluded as a factor of the outcome after pollen storage. Pollen performance *in vivo* is also influenced by pistil tissue (Hedhly *et al.*, 2005; Radičević *et al.*, 2016; Radunić *et al.*, 2017), and the comparing pollination experiment is possible only in two different seasons (with fresh pollen – in one season, and with stored pollen – in next flowering season), so the impact of female tissue in different seasons would also not be able to exclude as a factor contributing the outcome of pollen performance after storage.

This work was undertaken primarily to determine the influence of cryotreatment on pollen reproductive ability *in vivo*, compared to the performance of fresh pollen from the same season, to explore the influence of the freezing and thawing independently of the storage longevity. The influence of cryotreatment on pollen performance was considered from the context of its interaction with polliniser genotype, and the possible use of obtained findings for the future testing of pollen fertilization ability after short or long-term preservation. An important element of the working hypothesis was also that the key parameters of *in vivo* pollen performance, which show differences after freezing and storage within only one hour, can also be key elements of pollen evaluation after a longer period of storage.

MATERIALS AND METHODS

Plant Material, Growing Conditions and Experiment Design

The experiment was conducted in a sweet cherry orchard at 'Preljina' experimental site of the Fruit Research Institute, Čačak, West Serbia (43°53' N; 20°21' E; 350 m above the sea), over the 2019 season.

Pollen of cultivars 'Kordia' and 'Summit' was used for cryotreatment and pollination ('male' cultivars), whereas 'Regina' was a pollen recipient ('female' cultivar). The cultivars are self-incompatible, with the *S*-allelic constitutions S_3S_6, S_1S_2 and S_1S_3 respectively, which were confirmed in previous studies (Radičević *et al.*, 2013a; Schuster, 2017), so both pollination combinations ('Regina' × 'Kordia' and 'Regina' × 'Summit') were semi-compatible.

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

Pollen Collection and Cryotreatment

Approximately 50 flowers in the late balloon stage (growth stage 60, according to the BBCH scale reported by Meier, 2018) of both pollinisers were taken from all sides of cherry trees. The anthers were collected and kept in paper boxes in laboratory conditions for 48 hours at a temperature of 20ºC until they released pollen grains. Pollen was divided into three groups: i) samples intended for determination of pollen MC (approximately 400 mg per each replication), which was determined from pollen fresh weight and constant dry weight, obtained by oven-drying at 105°C for 45 min (Memmert GmbH + Co.KG, Büchenbach, Germany); ii) pollen samples that were placed in 1.8 ml cryotubes and directly immersed into LN (Cryo Diffusion B2020, Lery, France) for one hour – then thawing was done in water bath at 38°C for 1 min, after which cryotreated pollen was used for *in vitro* and *in vivo* testing; iii) fresh pollen samples.

Pollen Germination *In vitro*

Pollen of each treatment/polliniser combination was plated in three Petri dishes on nutrition medium (1% agar and 12% sucrose) and incubated at 20°C temperature for 24 hours. The germinated pollen grains were counted in three fields of view, each including about 100 pollen grains, using a microscope Olympus BX61 (light regime) and AnalySIS software (Olympus, Tokyo, Japan). As germinated pollen grains were considered those with tubes

exceeding their radius; pollen tube length was measured as well. Pollen germination percentage and pollen tube length were calculated as the average of nine microscopic observation fields.

Pollination Procedure

Two-year-old branches of 'Regina' with a uniform population of flowers (late balloon stage; BBCH 60) were chosen. Selected branches contained about 80–100 flowers; in this manner, about 4,000 flowers were selected (1,000 per treatment/polliniser combination), emasculated and protected with paper bags.

Pollination of emasculated flowers was done at the beginning of full flowering when stigmatic secretion was evident (BBCH 65). Approximately equal amounts of pollen were ensured with two touches of stigma (Winsor and Stephenson, 1995), providing an abundance of pollen. After hand-pollination, branches were isolated again with protective bags, which were permanently removed three weeks following the pollination.

Pollen Performance *In vivo* – Microscopic Observation and Fruit Set

A total of 100 pistils per treatment/polliniser combination was fixed 72, 144 and 240 hours after pollination in FPA (70% ethanol, propionic acid and formaldehyde, 90:5:5 percentages by volume). The aniline blue was used (Preil, 1970; Kho and Baër, 1971) to stain callose in the pollen tubes' inner layer, which permitted their clear visualization under ultra-violet light. The styles were opened along the suture, covered with a husk and squashed. The ovaries were dissected along the suture, and integuments of the primary ovules were cut with a razor blade longitudinally-tangentially, to enable better observation of pollen tube penetration in the micropyle and nucellus.

The pistils were observed under ultraviolet (UV) light on the Olympus BX61 microscope by AnalySIS software (Olympus, Tokyo, Japan), using Multiple Image Analysis (MIA). The pistils with good stigma receptivity were considered those with more than 20 pollen tubes in the upper third of the style, and they were included in further examination. Pollen tubes were counted in the upper third, middle third and the base of the style as well as in the ovary, at magnification of 200× (style parts) and 100× (ovary). The percentage of pistils with the longest tubes penetrating to pistils' particular parts per specific fixation term, was considered as the rate of the pollen tube growth dynamics. The total of pistils with penetration of pollen tube into the nucellus 240 hours after pollination was taken as the fertilization percentage.

At the beginning of ripening (BBCH 85), the fruit set was recorded, as the percentage of fruits per total number of pollinated flowers remaining after the final fixation.

Statistical Analysis

Two-factor analysis of variance (ANOVA) was used for data processing. Duncan's Multiple Range Tests at $P \leq 0.05$ were used for the determination of the significance of differences among mean values. Statistical analyses were performed using the SPSS statistical software package, Version 8.0 for Windows (SPSS. Inc., Chicago, IL).

RESULTS

Pollen MC measuring has not shown marked differences between pollinisers – 7.13% in 'Summit' and 7.48% in 'Kordia'. Cryotreatment did not influence pollen germination *in vitro*, but significantly influenced pollen tube length, i.e. significantly lower values were obtained in cryotreated pollen (269.72 µm) (269.72 µm) (Tab. 1; Fig. 1a), compared to fresh pollen (353.83 µm) (Tab. 1; Fig. 1b).

Table 1. *In vitro* performance of cryotreated/fresh sweet cherry pollen.

Factor		Pollen germination (%)	Pollen tube length (µm)
Treatment (A)	C	38.62±3.11	269.72±14.74[b]
	F	39.35±2.35	353.83±19.97[a]
Genotype (B)	'Kordia'	48.32±1.16[a]	302.54±23.70
	'Summit'	29.59±0.72[b]	321.01±24.84
A × B	C/'Kordia'	48.85±2.31	264.51±15.99
	C/'Summit'	28.39±0.59	274.92±28.34
	F/'Kordia'	47.91±1.13	340.56±33.23
	F/'Summit'	30.79±0.88	367.09±12.61
ANOVA	A	ns	**
	B	**	ns
	A × B	ns	ns

C – cryotreated pollen; F – fresh pollen

ns and ** indicate the level of significance at $P \leq 0.05$ and the absence of significance, respectively, according to Duncan's Multiple Range Test. Values are means ± standard error. Mean values followed by the different lower-case letters in the column represent significant differences.

On the other hand, pollen tube germination was significantly affected by genotype – the higher value of *in vitro* pollen germination was determined in 'Kordia' (48.32%) than in 'Summit' (29.59%); the differences in terms of pollen tube length by genotype were not observed.

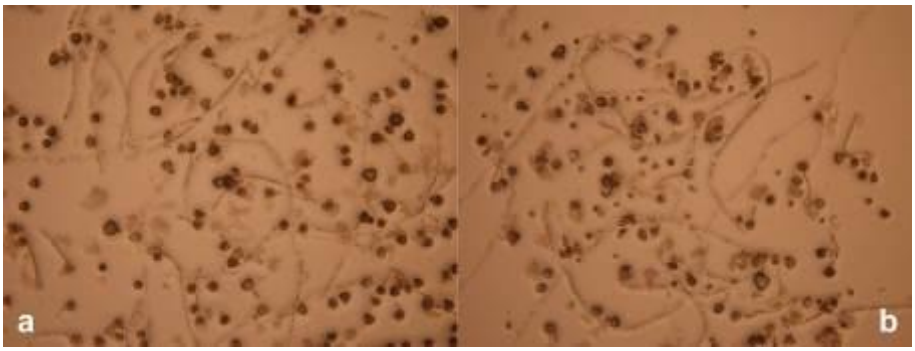

Figure 1. Pollen germination *in vitro* of 'Summit' sweet cherry: (a) cryotreated pollen; (b) fresh pollen.

A large number of pollen tubes in the upper third, and their drastic reduction in lower sections of the pistils, were observed in all treatment/polliniser combinations (Tab. 2; Fig. 2). Pollen tubes number in the upper part of the style, as well as in the ovary, was affected by cryotreatment, having significantly lower values for cryotreated (43.77 and 2.24, respectively), in comparison to fresh pollen (58.75 and 2.40, respectively) (Tab. 2).

Table 2. *In vivo* performance of cryotreated/fresh sweet cherry pollen.

Factor		Stu	Stm	Bs	Ovr	FP	FS
Treatment (A)	C	43.77±3.51^{b}	28.29±4.10	12.20±2.33	2.24±0.05^{b}	30.27±0.99	14.03±1.72
	F	58.75±8.41^{a}	26.32±7.57	9.99±1.51	2.40±0.05^{a}	29.52±2.84	15.72±3.79
Genotype (B)	'Kordia'	62.33±6.24^{a}	37.87±4.74^{a}	14.96±1.34^{a}	2.38±0.05	36.63±0.80^{a}	20.97±2.62^{a}
	'Summit'	40.21±4.21^{b}	16.73±2.71^{b}	7.22±0.63^{b}	2.26±0.05	22.89±1.15^{b}	9.04±1.22^{b}
A × B	C/'Kordia'	50.33±4.22	34.58±6.25	17.33±0.73	2.27±0.03	33.71±0.15^{b}	17.25±1.79
	C/'Summit'	37.20±0.72	22.00±2.22	7.06±0.35	2.22±0.10	26.82±0.71^{c}	10.80±1.83
	F/'Kordia'	74.33±5.75	41.17±7.90	12.58±1.67	2.48±0.01	39.54±0.14^{a}	24.14±5.13
	F/'Summit'	43.22±8.89	11.47±2.01	7.39±1.37	2.30±0.04	19.50±0.07^{d}	7.29±1.29
ANOVA	A	**	ns	ns	**	ns	ns
	B	**	**	**	ns	**	**
	A × B	ns	ns	ns	ns	**	ns

C – cryotreated pollen; F – fresh pollen; Stu – upper part of the style; Stm – middle part of the style; Bs – base of the style; Ovr – ovary; FP – fertilization percentage; FS – fruit set.

ns and ** indicate the level of significance at $P \leq 0.05$ and the absence of significance, respectively, according to Duncan's Multiple Range Test. Values are means ± standard error. Mean values followed by the different lower-case letters in the column represent significant differences.

Otherwise, pollen tubes number was affected by polliniser genotype in all parts of the style, with significantly higher values with 'Kordia' as polliniser. Pollen tubes number in the ovary was not influenced by polliniser genotype.

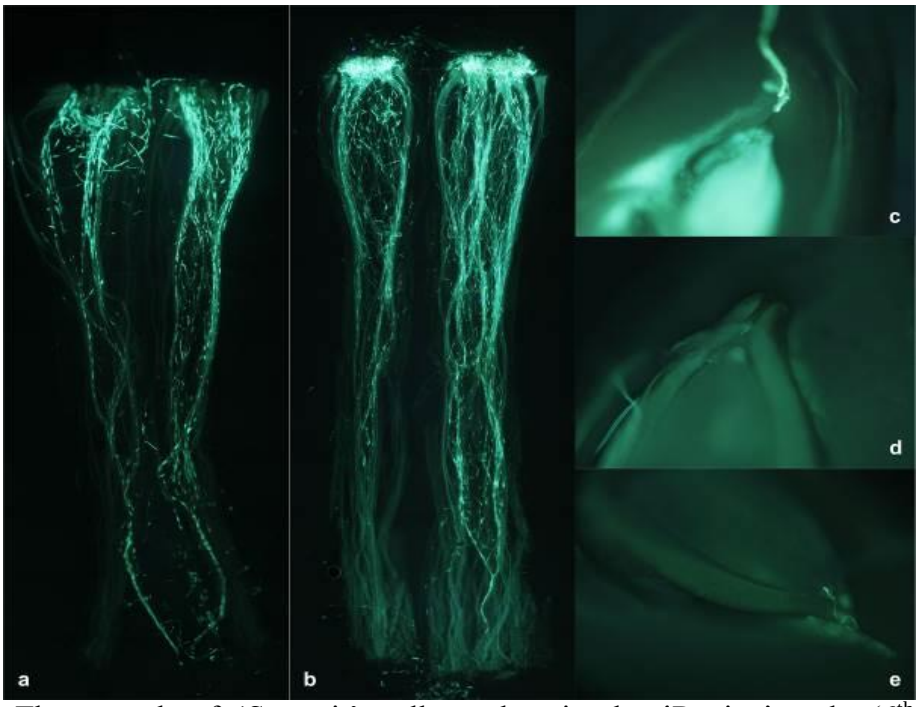

Figure 2. The growth of 'Summit' pollen tubes in the 'Regina' style (6th day after pollination): cryotreated pollen (a), fresh pollen (b); the growth of 'Summit' pollen tubes (cryotreated pollen) in the 'Regina' ovary – obturator area (c), micropyle (d), penetration into the nucellus (e).

Pollen tubes were observed in the ovary on the third day after pollination in all treatment/polliniser combinations (Fig. 3). The longest pollen tubes were located predominantly in the obturator zone, with a lower rate of occurrence in the micropyle (fresh pollen of 'Kordia'). On the sixth day after pollination, the pistils generally had the longest pollen tube in the nucellus, except cryotreated pollen of 'Kordia' (the longest pollen tube in the micropyle). On the tenth day, the longest pollen tubes were found in the nucellus, in all treatment/polliniser combinations.

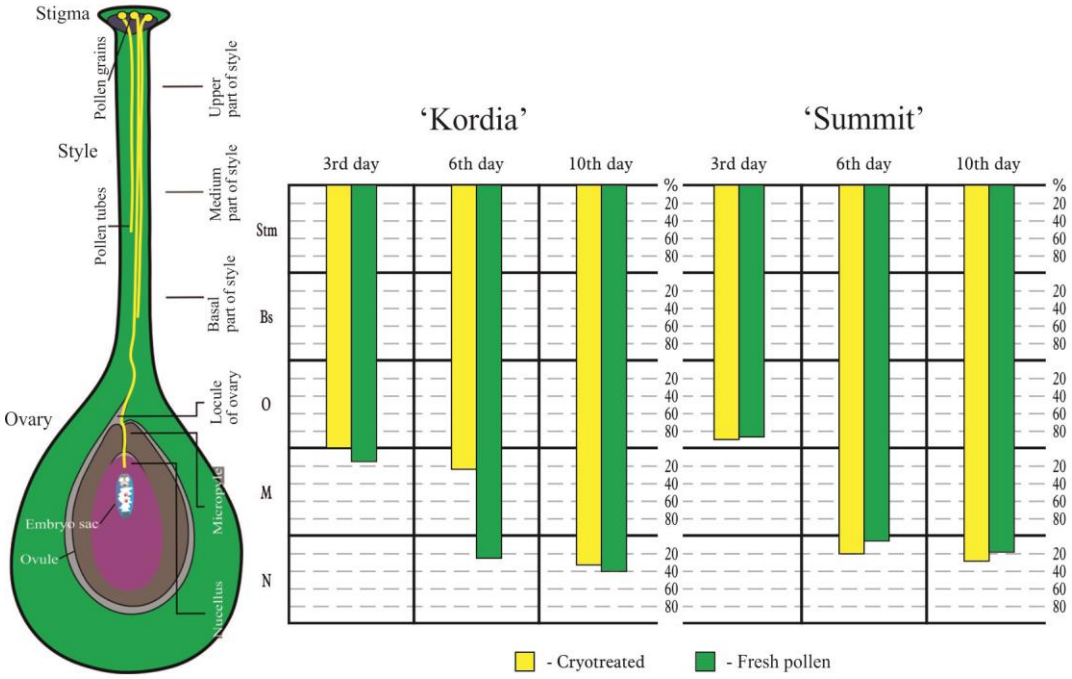

Figure 3. Dynamics of pollen tube growth *in vivo* in the pistils of 'Regina' after cryotreatment. Stm – middle part of the style; Bs – base of the style; O – obturator zone; M – micropile; N – nucellus of the ovule.

Although pollen tubes dynamics generally had good values for both pollinisers, treatments and fixation terms, it is noticeable that the values had opposite tendencies by pollinisers – for 'Kordia', they were slightly better for fresh pollen, whereas for 'Summit' they were even better when the pollen was cryotreated. Fertilization percentage was affected by polliniser genotype and interaction among the variability factors (Tab. 2), pointing to the fact that cryotreatment acted with unequal intensity on the behaviour of pollinisers. On the other hand, fruit set was influenced by polliniser genotype, having higher values with 'Kordia' (20.97%), in comparison to 'Summit' as polliniser (9.04%).

DISCUSSION

Pollen Performance and Impact of Cryotreatment

Factors affecting the viability of pollen during storage were the moisture content, as well as physiological stage of the flower and the age of the pollen (Čalić *et al.*, 2021). Pollen grains are susceptible to injury caused by rapid water uptake during rehydration, which can reduce germination. This 'imbibitional injury' can be aggravated by low temperatures due to mechanical damage of the plasmalemma, as polar lipids undergo phase changes as a result of temperature fluctuations, water and sugar content (Hoekstra and Van der Wal, 1988; Crowe *et al.*, 1989; Hoekstra *et al.*, 1992). In general, the reduction of pollen MC improves the success

of long-term conservation. However, pollen should fully tolerate the dehydration process (Hong *et al.*, 1999), which is related to pollen morphology. *Prunus* species have binucleate, developmentally immature pollen that, when dispersing from the anther, had reduced metabolic activity, low moisture content and could better tolerate desiccation compared to trinucleate pollen (Franchi *et al.*, 2011). It can be dehydrated to low MC (between 5% and 10% on a fresh weight basis), stored at ultra-low temperatures (in LN) and successfully thawed at room temperature (Dinato *et al.*, 2020). Our results on pollen performance *in vitro* (Tab. 1) indicate that short-term cryotreatment of sweet cherry pollen dehydrated to MC slightly higher than 7% did not affect the physiological processes liable for pollen grains' germination ability. In other *Rosaceae* fruit species – almond (Martínez-Gómez et al., 2002), apple (Čalić et al., 2021) or plum (Đorđević et al., 2022) that has not been shown and this difference was primarily the result of cryotreatment longevity (pollen storage, quite longer than in this work). On the other hand, short-term cryotreatment applied in this work affected the initial strength of pollen tubes' growth *in vitro*, manifesting as an effect on their length.

This effect is also visible in terms of the number of pollen tubes in the pistil, primarily in its upper part, as well as in the ovary (Tab. 2). Our results indicate that pollen performance *in vivo* to some extent is a reflection of its behaviour *in vitro* in both cryotreated/fresh pollen, as it has been already shown for *Prunus* species pollen without cryotreatment (Đorđević *et al.*, 2012; Radičević *et al.*, 2016).

On the other hand, the influence of cryotreatment was not manifested in the pollen tube number in the middle third and the base of the style. Style length is, for many sweet cherry cultivars, from 12.5 mm to 14 mm (Zhang *et al.*, 2012), so the upper third length matches with the pollen tubes length *in vitro* – even though stigma provides a rich germination environment, and stylar tissue is more supportive for pollen tube growth than *in vitro* medium. It seems that the pollen tube number, which is in specific style parts comparable to pollen tube length, was influenced by cryotreatment below the stigma and in the upper stylar part. With the distance from the stigma, pollen tube numbers were less dependent on cryotreatment and more dependent on genotype, gametophyte-sporophyte interactions, and other possible factors that contribute to the interaction.

The impact of cryotreatment on the outcome of the fertilization process, expressed through fertilization percentage and especially fruit set did not manifest itself, which is encouraging given the possibility of using frozen and reverse thawed pollen for breeding and other

purposes, leading to the conclusion that these procedures, generally, do not impair the reproductive ability of sweet cherry pollen.

Pollen Performance and Impact of Polliniser Genotype

Opposite the effect of cryotreatment, the polliniser genotype influenced pollen germination but did not influence pollen tube length *in vitro* (Tab. 1). 'Kordia' had significantly better *in vitro* pollen germination independently of cryotreatment; as for *in vivo* performance, this cultivar also induced higher number of pollen tubes along the style (Tab. 2).

Regularity in microsporogenesis is directly associated with vitality and *in vitro* pollen germination (Radičević *et al.*, 2013b). In our experiment, this factor overcoming has been achieved through an experiment setting, which allows comparing the performance of cryotreated/fresh pollen from the same season. In addition, these tendencies may also be due to the experimental conditions, such as incubation temperature and composition of the medium.

At first sight, no big differences among pollinisers' behaviour were observed during the pollen tube growth dynamics monitoring, considering that on the tenth day after pollination, the pollen tubes of both pollinisers were in the nucellus (Fig. 3). It is noticeable that pollen tubes of 'Kordia' had somewhat better dynamics on the third day after pollination than pollen tubes of 'Summit'. The value of fertilization percentage and fruit set were also higher with 'Kordia', confirming previous findings that better polliniser efficacy has been achieved primarily through the pollen tube number, i.e. a higher number of pollen tubes gives preconditions for better efficiency in terms of penetration into the nucellus (Radičević *et al.*, 2016).

Pollen Performance and Cryotreatment/Polliniser Interaction

Pollen tube growth rate in 'Kordia' had lower values by fixation terms in the cryotreated variant, whereas in 'Summit' in the same variant had higher values (Fig. 3). Cryotreatment has modified the reproductive behaviour of pollinisers in different ways and different strength, resulting in significance of variability factors' interaction on the fertilization percentage (Tab. 2). Some kind of uneven influence of cryotreatment on behaviour of different pollinisers, has also been shown in plum (Đorđević *et al.*, 2022).

The total effect of this interaction has surely included the effect of female genotype on polliniser behaviour, although the choice of pollinated cultivar was made to minimize this effect – none of the pollinisers was less or more supported by stylar tissue of 'Regina' (both

combinations are semi-compatible). ‘Regina’ is known as a cultivar with a low level of ovule senescence, supporting pollen tube growth in the ovary (Radičević *et al.*, 2018). Besides these facts, the other kinds of male/female interactions have also contributed to the summarized interaction effect. They include air temperature impacts before and during the flowering, which were also reported (Radičević *et al.*, 2016); the authors pointed out that the reproductive behaviour of ‘Kordia’ as an autochthonous genotype from the northern Czech Republic is a reflection of its geographic origin. Orlova *et al.* (2019) reported that parameters of pollen performance *in vitro* after cryopreservation in autochthonous sweet cherry cultivars have been related to their geographic origin.

CONCLUSIONS

Pollen preservation should be implemented as a standard procedure for breeding purposes and biodiversity conservation in genebanks, as well as developing handling and assessing procedures for different fruit species and cultivars. Our results indicate that its reproductive ability is verifiable by analysing *in vivo* parameters, which should be combined with *in vitro* tests. Considering all the parameters of *in vitro*/*in vivo* performance of sweet cherry pollen after short-term cryotreatment in comparison to the fresh pollen from the same season, clearly indicates the way that cryotreatment and thawing influence pollen reproductive ability, independently of storage longevity. For this kind of testing, semi- or fully-compatible female recipients, whose sporophytes are supportive of certain male gametophytes, should be used. The key parameters of *in vivo* testing that should be in focus are pollen tube number in the upper part of the style, pollen tube number in the ovary, as well and fertilization percentage, i.e. the percentage of pistils with penetration of the longest pollen tube in the nucellus of the ovary on the 10^{th} day after pollination. Controlling these parameters is crucial through the procedure of checking the reproductive ability of stored pollen, due to the difference in comparison to the fresh pollen occurs even at the short-term storage. This way is relatively simple and significantly shorter than monitoring fruit set and seed production, giving the answers about pollen quality with valuable credibility, in maximum of two weeks after thawing and rehydration.

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