ACCEPTED ARTICLE

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

Running title: Sweet Cherry Pollen Performance after Cryotreatment

S. Radičević^{1*}, T. Vujović², S. Marić¹, N. Milošević¹, I. S. Glišić¹, M. Đorđević¹, T. Anđelić

11 ABSTRACT

1 2

3 4

5

6 7

8

9 10

12 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 13 L.) pistils. The influence of cryotreatment was considered from the context of its interaction 14 15 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 16 cryotreated and fresh pollen of 'Kordia' and 'Summit'. Quantitative parameters of pollen tube 17 18 growth in vivo (pollen tube number in certain pistil parts, pollen tube dynamics and fertilization percentage) were determined using the fluorescence microscopy method, and 19 20 obtained data were analysed together with those obtained for in vitro pollen performance and 21 fruit set. The research has indicated that: i) the reproductive ability of cryotreated sweet 22 cherry pollen can be estimated by analysing parameters of its performance in vivo, which 23 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 24 25 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 26 the longest pollen tube in the nucellus of the ovary on the 10th day after pollination. Therefore, 27 28 *in vivo* testing of the pollen reproductive ability after cryotreatment can give answers about 29 pollen quality with valuable credibility, in a maximum of two weeks after thawing and 30 rehydration.

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

¹Department of Pomology and Fruit Breeding, Fruit Research Institute, Čačak, Kralja Petra I 9, 32000 Čačak Republic of Serbia (Sanja Radičević, Slađana Marić, Nebojša Milošević, Ivana Glišić, Milena Đorđević). ²Department of Fruit Physiology, Fruit Research Institute, Čačak, Kralja Petra I 9, 32000 Čačak, Republic of Serbia (Tatjana Vujović, Tatjana Anđelić).

^{*}Corresponding author; e-mail: sradicevic@institut-cacak.org

34 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).

40 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 41 42 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 43 44 pollen metabolism decrease, which allows greater longevity and viability independent of the 45 storage period (Dinato et al., 2020). Long-term storage of fruit species pollen makes cross-46 breeding possible between the cultivars differing in flowering time, or between plants 47 growing in remote places, resulting in extended utilization of germplasm.

48 Cryopreservation is a simple and highly efficient method used to conserve plant genepool 49 components - seeds, tissue material, etc. (Engelmann, 2004; Ružić et al., 2014; Vujović et al., 50 2015), as well as pollen grains (Rajasekharan et al., 2013; Dinato et al., 2020). The mature 51 male gametophyte of most plant species is desiccation tolerant, which renders them ideally 52 suitable for storage, especially under cryogenic conditions (Rajasekharan et al., 2013). 53 Moisture content (MC) plays a major role during cryopreservation – excess moisture in pollen 54 forms ice crystals damaging pollen membranes and causing alterations in the structural 55 integrity of cells, resulting in mechanical and physical injury that ultimately leads to viability 56 loss (Xu et al., 2014). Unlike multicellular plant structures in which extracellular ice 57 formation reduces the possibility of the cytoplasm freezing, pollen grains are single entities 58 and this mechanism of protection is likely limited (Dinato et al., 2020). Thus, the hydration 59 level must be decreased before cooling and according to Connor and Towill (1993) the MC of 60 the pollen intended for successful long-term conservation at -80°C to -196°C should be between 7% and 20%. 61

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 (-196° C), 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 70 71 cryopreserved pollen for breeding and other purposes in heterozygous and polyploid fruit tree 72 species. Up to now, many pollen viability and germinability tests in vitro have been used 73 (Martínez-Gómez et al., 2002; Duta et al., 2013; Čalić et al., 2021), with changeable 74 conditions of testing (pollen rehydration, staining, medium content, etc). More recent results, 75 obtained in plums (Dorđević et al., 2022), deal with the pollen performance in vivo after a certain period of storage. These investigations focused on pollen storage longevity itself, 76 77 giving us valuable data related to the possibility of after-storage usage, but without the 78 possibility to compare pollen performance, particularly in vivo, of cryotreated and fresh pollen 79 from the same season; the only possible is comparing of one-year-stored pollen with the fresh 80 pollen from next season, due to biological cycle. In that way, the influences of 81 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 82 83 performance in vivo is also influenced by pistil tissue (Hedhly et al., 2005; Radičević et al., 84 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 85 86 flowering season), so the impact of female tissue in different seasons would also not be able 87 to exclude as a factor contributing the outcome of pollen performance after storage.

88 This work was undertaken primarily to determine the influence of cryotreatment on pollen 89 reproductive ability in vivo, compared to the performance of fresh pollen from the same 90 season, to explore the influence of the freezing and thawing independently of the storage 91 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 92 for the future testing of pollen fertilization ability after short or long-term preservation. An 93 94 important element of the working hypothesis was also that the key parameters of *in vivo* 95 pollen performance, which show differences after freezing and storage within only one hour, 96 can also be key elements of pollen evaluation after a longer period of storage.

98 MATERIALS AND METHODS

99 Plant Material, Growing Conditions and Experiment Design

97

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 semicompatible.

109 All three cultivars (pollinisers and pollen recipient) were grafted on the 'Gisela 5' rootstock, 110 and the orchard was established in the spring of 2005, with a spacing of 4.0×1.5 m. The 111 Zahn Spindle training system was used. Standard cultural practices (pruning, fertilization, drip 112 irrigation, pest and disease control) were included within orchard management. A randomized 113 block design was used for the experiment set-up, with three replications (three trees each).

114

115 **Pollen Collection and Cryotreatment**

116 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. 117 118 The anthers were collected and kept in paper boxes in laboratory conditions for 48 hours at a 119 temperature of 20°C until they released pollen grains. Pollen was divided into three groups: i) 120 samples intended for determination of pollen MC (approximately 400 mg per each 121 replication), which was determined from pollen fresh weight and constant dry weight, 122 obtained by oven-drying at 105°C for 45 min (Memmert GmbH + Co.KG, Büchenbach, 123 Germany); ii) pollen samples that were placed in 1.8 ml cryotubes and directly immersed into 124 LN (Cryo Diffusion B2020, Lery, France) for one hour – then thawing was done in water bath 125 at 38°C for 1 min, after which cryotreated pollen was used for *in vitro* and *in vivo* testing; iii) 126 fresh pollen samples.

127

128 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.

137

138 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.

148

149 Pollen Performance In vivo – Microscopic Observation and Fruit Set

150 A total of 100 pistils per treatment/polliniser combination was fixed 72, 144 and 240 hours 151 after pollination in FPA (70% ethanol, propionic acid and formaldehyde, 90:5:5 percentages 152 by volume). The aniline blue was used (Preil, 1970; Kho and Baër, 1971) to stain callose in 153 the pollen tubes' inner layer, which permitted their clear visualization under ultra-violet light. 154 The styles were opened along the suture, covered with a husk and squashed. The ovaries were 155 dissected along the suture, and integuments of the primary ovules were cut with a razor blade 156 longitudinally-tangentially, to enable better observation of pollen tube penetration in the 157 micropyle and nucellus.

158 The pistils were observed under ultraviolet (UV) light on the Olympus BX61 microscope by 159 AnalySIS software (Olympus, Tokyo, Japan), using Multiple Image Analysis (MIA). The 160 pistils with good stigma receptivity were considered those with more than 20 pollen tubes in 161 the upper third of the style, and they were included in further examination. Pollen tubes were 162 counted in the upper third, middle third and the base of the style as well as in the ovary, at 163 magnification of $200 \times$ (style parts) and $100 \times$ (ovary). The percentage of pistils with the longest tubes penetrating to pistils' particular parts per specific fixation term, was considered 164 165 as the rate of the pollen tube growth dynamics. The total of pistils with penetration of pollen 166 tube into the nucellus 240 hours after pollination was taken as the fertilization percentage.

167 At the beginning of ripening (BBCH 85), the fruit set was recorded, as the percentage of 168 fruits per total number of pollinated flowers remaining after the final fixation.

169

170 Statistical Analysis

Two-factor analysis of variance (ANOVA) was used for data processing. Duncan's Multiple Range Tests at $P \le 0.05$ 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).

- 175
- 176

177 **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 μ m) (269.72 μ m) (Tab. 1; Fig. 1a), compared to fresh pollen (353.83 μ m) (Tab. 1; Fig. 1b).

183 184

185

186

187

188

189

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

	1	2	<u> </u>	
Factor		Pollen germination	Pollen tube length	
		(%)	(µm)	
$T_{restment}(\Lambda)$	С	38.62±3.11	269.72±14.74 ^b	
Treatment (A)	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	
$A \times B$	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	А	ns	**	
	В	**	ns	
	$\mathbf{A} \times \mathbf{B}$	ns	ns	

C – cryotreated pollen; F – fresh pollen

ns and ** indicate the level of significance at $P \le 0.05$ and the absence of significance, respectively, according to Duncan's Multiple Range Test. Values are means \pm 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.



194

195 Figure 1. Pollen germination *in vitro* of 'Summit' sweet cherry: (a) cryotreated pollen; (b) 196 fresh pollen.

197

198 A large number of pollen tubes in the upper third, and their drastic reduction in lower 199 sections of the pistils, were observed in all treatment/polliniser combinations (Tab. 2; Fig. 2). 200 Pollen tubes number in the upper part of the style, as well as in the ovary, was affected by 201 cryotreatment, having significantly lower values for cryotreated (43.77 and 2.24, respectively), 202 in comparison to fresh pollen (58.75 and 2.40, respectively) (Tab. 2).

203

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

204 Table 2. <i>In vivo</i> performance of cryotreated/fresh sweet cherry pollen.								
F	Factor	Stu	Stm	Bs	Ovr	FP	FS	
Treatment	С	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	
(A)	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	'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	
(B)	'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	
$\mathbf{A} \times \mathbf{B}$	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°	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	А	**	ns	ns	**	ns	ns	
	В	**	**	**	ns	**	**	
	$\mathbf{A} \times \mathbf{B}$	ns	ns	ns	ns	**	ns	

205C – 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. 206

207 ns and ** indicate the level of significance at $P \le 0.05$ and the absence of significance, respectively, according 208 to Duncan's Multiple Range Test. Values are means \pm standard error. Mean values followed by the different 209 lower-case letters in the column represent significant differences. 210

211 Otherwise, pollen tubes number was affected by polliniser genotype in all parts of the style,

212 with significantly higher values with 'Kordia' as polliniser. Pollen tubes number in the ovary

213 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).

219

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.



227

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.

232 Although pollen tubes dynamics generally had good values for both pollinisers, treatments 233 and fixation terms, it is noticeable that the values had opposite tendencies by pollinisers – for 234 'Kordia', they were slightly better for fresh pollen, whereas for 'Summit' they were even 235 better when the pollen was cryotreated. Fertilization percentage was affected by polliniser 236 genotype and interaction among the variability factors (Tab. 2), pointing to the fact that 237 cryotreatment acted with unequal intensity on the behaviour of pollinisers. On the other 238 hand, fruit set was influenced by polliniser genotype, having higher values with 'Kordia' (20.97%), in comparison to 'Summit' as polliniser (9.04%). 239

240 **DISCUSSION**

241 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 249 250 (Hong et al., 1999), which is related to pollen morphology. Prunus species have binucleate, 251 developmentally immature pollen that, when dispersing from the anther, had reduced 252 metabolic activity, low moisture content and could better tolerate desiccation compared to 253 trinucleate pollen (Franchi et al., 2011). It can be dehydrated to low MC (between 5% and 254 10% on a fresh weight basis), stored at ultra-low temperatures (in LN) and successfully 255 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 256 257 slightly higher than 7% did not affect the physiological processes liable for pollen grains' 258 germination ability. In other Rosaceae fruit species - almond (Martínez-Gómez et al., 2002), 259 apple (Čalić et al., 2021) or plum (Đorđević et al., 2022) that has not been shown and this 260 difference was primarily the result of cryotreatment longevity (pollen storage, quite longer than 261 in this work). On the other hand, short-term cryotreatment applied in this work affected the 262 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).

268 On the other hand, the influence of cryotreatment was not manifested in the pollen tube 269 number in the middle third and the base of the style. Style length is, for many sweet cherry 270 cultivars, from 12.5 mm to 14 mm (Zhang et al., 2012), so the upper third length matches with 271 the pollen tubes length in vitro - even though stigma provides a rich germination environment, 272 and stylar tissue is more supportive for pollen tube growth than in vitro medium. It seems that 273 the pollen tube number, which is in specific style parts comparable to pollen tube length, was 274 influenced by cryotreatment below the stigma and in the upper stylar part. With the distance 275 from the stigma, pollen tube numbers were less dependent on cryotreatment and more 276 dependent on genotype, gametophyte-sporophyte interactions, and other possible factors that 277 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 thereproductive ability of sweet cherry pollen.

283

284 **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.

295 At first sight, no big differences among pollinisers' behaviour were observed during the 296 pollen tube growth dynamics monitoring, considering that on the tenth day after pollination, 297 the pollen tubes of both pollinisers were in the nucellus (Fig. 3). It is noticeable that pollen 298 tubes of 'Kordia' had somewhat better dynamics on the third day after pollination than pollen 299 tubes of 'Summit'. The value of fertilization percentage and fruit set were also higher with 300 'Kordia', confirming previous findings that better polliniser efficacy has been achieved 301 primarily through the pollen tube number, i.e. a higher number of pollen tubes gives 302 preconditions for better efficiency in terms of penetration into the nucellus (Radičević et al., 303 2016).

304

305 **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 315 combinations are semi-compatible). 'Regina' is known as a cultivar with a low level of ovule 316 senescence, supporting pollen tube growth in the ovary (Radičević et al., 2018). Besides these 317 facts, the other kinds of male/female interactions have also contributed to the summarized 318 interaction effect. They include air temperature impacts before and during the flowering, 319 which were also reported (Radičević et al., 2016); the authors pointed out that the 320 reproductive behaviour of 'Kordia' as an autochthonous genotype from the northern Czech 321 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 322 323 have been related to their geographic origin.

324

325 CONCLUSIONS

326 Pollen preservation should be implemented as a standard procedure for breeding purposes 327 and biodiversity conservation in genebanks, as well as developing handling and assessing 328 procedures for different fruit species and cultivars. Our results indicate that its reproductive 329 ability is verifiable by analysing *in vivo* parameters, which should be combined with *in vitro* 330 tests. Considering all the parameters of *in vitro/in vivo* performance of sweet cherry pollen 331 after short-term cryotreatment in comparison to the fresh pollen from the same season, clearly 332 indicates the way that cryotreatment and thawing influence pollen reproductive ability, 333 independently of storage longevity. For this kind of testing, semi- or fully-compatible female 334 recipients, whose sporophytes are supportive of certain male gametophytes, should be used. 335 The key parameters of *in vivo* testing that should be in focus are pollen tube number in the 336 upper part of the style, pollen tube number in the ovary, as well and fertilization percentage, 337 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. Controlling these parameters is crucial through the 338 339 procedure of checking the reproductive ability of stored pollen, due to the difference in 340 comparison to the fresh pollen occurs even at the short-term storage. This way is relatively 341 simple and significantly shorter than monitoring fruit set and seed production, giving the 342 answers about pollen quality with valuable credibility, in maximum of two weeks after 343 thawing and rehydration.

345 ACKNOWLEDGEMENTS

This research was conducted under the support of the Ministry of Science, Technological Development and Innovations of the Republic of Serbia, contract No. 451-03-47/2023-01/200215 (including project No. 31064, titled 'Development and preservation of genetic

344

349 potential of temperate zone fruits').

350

351 **REFERENCES**

Cerović, R. 1991. Cytogenetic Properties of Sour Cherry in Relation to Pollen.
 Genetika-Belgrade, 23: 247–258.

2. Connor, O. F. and Towill, L. E. 1993. Pollen-Handling Protocol and
Hydration/Dehydration Characteristics of Pollen for Application to Long-term Storage. *Euphytica*, 68: 77–84.

357 3. Crowe, J. H., Crowe, L. M., Hoekstra, F. A. and Wistrom, C. A. 1989. Effects of 358 Water on the Stability of Phospholipid Bilayers: The Problem of Imbibition Damage in Dry 359 Organisms. In: *"Seed Moisture*", Special Publication no 14., (Eds.): Stanwood, P. C. and 360 McDonald, M. B., Crop Science Society of America Inc., USA, PP. 1–14.

361 4. Čalić, D., Milojević, J., Belić, M., Miletić, R. and Zdravković Korać, S. 2021. Impact
362 of Storage Temperature on Pollen Viability and Germinability of Four Serbian Autochthon
363 Apple Cultivars. *Front. Plant Sci.*, **12**: 709231.

5. Dinato, N. B., Imaculada Santos, I. R., Zanotto Vigna, B. B., De Paula, A. F. and
Fávero, A. P. 2020. Pollen Cryopreservation for Plant Breeding and Genetic Resources
Conservation. *Cryo-Lett.*, 41: 15–127.

367 6. Dutta, S. K., Srivastav, M., Chaudhary, R., Lal, K., Patil, P., Singh, S. K. and Sing, A.
368 K. 2013. Low Temperature Storage of Mango (*Mangifera inidica* L.) Pollen. *Sci. Hortic.*,
369 161: 193–197.

370 7. Đorđević., M., Radičević, S., Cerović, R., Milošević, N. and Mitrović, M. 2012. Initial
371 and Final Fruit Set in Plum Cultivar 'Pozna Plava' as Affected by Different Types of
372 Pollination. *Acta Hortic.*, 968: 121–124.

8. Đorđević, M., Vujović, T., Cerović, R., Glišić, I. S., Milošević, N., Marić, S., Radičević, S., Fotirić Akšić M. and Meland M. 2022. *In vitro* and *in vivo* Performance of Plum (*Prunus domestica* L.) Pollen from the Anthers Stored at Distinct Temperatures for Different Periods. *Horticulturae*, **8:** 616.

377 9. Engelmann, F. 2004. Plant Cryopreservation: Progress and Prospects. *In Vitro Cell.*378 *Dev. Biol. Plant*, 40: 427–433.

379 10. Franchi, G. G., Piotto, B., Nepi, M., Baskin, C. C., Baskin, J. M. and Pacini, E. 2011.
380 Pollen and Seed Desiccation Tolerance in Relation to Degree of Developmental Arrest,
381 Dispersal and Survival. *J. Exp. Bot.*, 62: 5267–5281.

- 382 11. Giovannini, A., Macovei, A., Caser, M., Mansuino, A., Ghione, G. G., Savona, M.,
 383 Carbonera, D., Scariot, V. and Balestrazzi, A. 2017. Pollen Grain Preservation and Fertility in
 384 Valuable Commercial Rose Cultivars. *Plants*, 6: 17.
- 12. Hedhly, A., Hormaza J. I. and Herrero, M. 2005. Influence of Genotype-Temperature
 Interaction on Pollen Performance. *J. Evol. Biol.*, 18: 1494–1502.
- 13. Hoekstra, F. A., Crowe, J. H. and Crowe, L. M. 1992. Germination And Ion Leakage
 are Linked with Phase Transitions of Membrane Lipids During Imbibition of *Typha Latifolia*L. Pollen. *Physiol. Plantarum*, 84: 29–34.
- Hoekstra, F. A. and Van der Wal, E. G. 1988. Initial Moisture Content and
 Temperature of Imbibition Determine Extent of Imbibitional Injury in Pollen. *J. Plant Physiol.*, 133: 257–262.
- Hong, T. D., Ellis, R. H., Buitink, J., Walters, C., Hoekstra, F. A. and Crane, J. 1999. A
 Model of the Effect of Temperature and Moisture on Pollen Longevity in Air-dry Storage
 Environments. *Ann. Bot.-London*, 83: 167–173.
- Kho, Y. O. and Baër, J. 1971. Fluorescence Microscopy in Botanical Research. *Zeiss- Inf.*, **76:** 54–57.
- 398 17. Martínez-Gómez, P., Gradziel, T. M., Ortega, E. and Dicenta, F. 2002. Low
 399 Temperature Storage of Almond Pollen. *HortScience*, 37: 691–692.
- 400 18. Meier, U. 2018. Growth stages of mono- and dicotyledonous plants: BBCH
 401 Monograph. Julius Kühn-Institut (JKI), Germany.
- 402 19. Orlova, S. Y., Pavlov, A. and Verzhuk, V. G. 2019. Viability of Pollen in Sweet
 403 Cherry (*Cerasus avium*) Varieties of Different Ecogeographic Origin in The Northwestern
 404 Region of Russia. *Proceedings on Applied Botany, Genetics and Breeding*, 180: 66–72. (*in*405 *Russian*).
- 20. Panella, L., Wheeler, L. and McClintock, M. E. 2009. Long-Term Survival of
 Cryopreserved Sugarbeet Pollen. *J. Sugar Beet Res.*, 46: 1–9.
- Preil, W. 1970. Observing of Pollen Tube in Pistil and Ovarian Tissue by Means of
 Fluorescence Microscopy. *Zeiss-Inf.*, **75**: 24–25.
- 22. Radičević, S., Cerović, R. and Đorđević, M. 2018. Ovule Senescence and Unusual
 Pollen Tube Growth in The Ovary of Sweet Cherry as Affected by Pistilar Genotype and
 Temperature. Span. J. Agric. Res., 16: e0704.

23. Radičević, S., Cerović, R., Nikolić, D. and Đorđević, M. 2016. The Effect of
Genotype and Temperature on Pollen Tube Growth and Fertilization in Sweet Cherry (*Prunus avium* L.). *Euphytica*, 209: 121–136.

416 24. Radičević, S., Marić, S., Cerović, R. and Đorđević, M. 2013a. Assessment of Self417 (In)Compatibility in Some Sweet Cherry (Prunus avium L.) Genotypes. Genetika-Belgrade,
418 45: 939–952.

419 25. Radičević, S., Nikolić, D., Cerović, R. and Đorđević, M. 2013b. *In vitro* Pollen
420 Germination and Pollen Grain Morphology in some Sweet Cherry (*Prunus avium* L.)
421 Cultivars. *Rom. Biotech. Lett.*, 18: 8341–8349.

422 26. Radunic, M., Jazbec, A., Ercisli, S., Čmelik, Z. and Goreta Ban, S. 2017. Pollen-pistil
423 Interaction Influence on the Fruit Set of Sweet Cherry. *Hortic. Sci.*, 224: 358–366.

424 27. Rajasekharan, P. E., Ravish, B. S., Vasantha Kumar, T. and Ganeshan, S. 2013. Pollen

425 Cryobanking for Tropical Plant Species. In: "Conservation of Tropical Plant Species", (Eds.):

426 Norma, M. N., Chin, H. F. and Reed, B. M. Springer-Verlag, New York, USA, PP. 1–12.

427 28. Rajasekharan, P. E. and Rohini, M. R. 2023. Pollen Cryopreservation: Advances and

428 Prospects. In: "Pollen Cryopreservation Protocols", (Eds.): Rajasekharan, P. E. and Rohini,

429 M. R. Springer Protocols Handbooks, Humana, New York, USA, PP. 1–18.

430 29. Ružić, Dj., Vujović, T. and Cerović, R. 2014. Cryopreservation of Cherry Rootstock
431 Gisela 5 Using Vitrification Procedure. *Hort. Sci. (Prague)*, 41: 55–63.

432 30. Schuster, M. 2017. Self-Incompatibility (*S*) Genotypes of Cultivated Sweet Cherries –
433 an Overview 2017. Quedlinburg 2017. OpenAgrar-Repositorium.

434 31. Sparks, D. and Yates, I. E. 2002. Pecan Pollen Stored Over a Decade Retains
435 Viability. *Hortic. Sci.*, 37: 176–177.

436 32. Vujović, T., Chatelet, P., Ružić, Đ. and Engelmann, F. 2015. Cryopreservation of
437 *Prunus* spp. Using Aluminium Cryo-plates. *Sci. Hortic-Amsterdam*, **195**: 173–182.

438 33. Winsor, J. A. and Stephenson, A. G. 1995. Demographics of Pollen Tube Growth in
439 *Cucurbita pepo. Can. J. Botany*, **73**: 583–589.

34. Xu, J., Li, B., Liu, Q., Shi, Y., Peng, J., Jia, M. and Liu Y. 2014. Wide-scale Pollen
Banking of Ornamental Plants through Cryopreservation. *CryoLetters*, 35: 312–319.

442 35. Zhang, L., Ampatzidis, Y. and Whiting, M. D. 2012. Sweet Cherry Floral Organ Size
443 Varies with Genotype and Temperature. *Sci. Hortic-Amsterdam*, 182: 156–164.

444

445