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In vitro POLLEN VIABILITY, GERMINATION AND POLLEN TUBE GROWTH IN SOME POMEGRANATE (*Punica granatum* L.) CULTIVARS FROM CROATIA AND BOSNIA AND HERZEGOVINA

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Abstract. In this study *in vitro* pollen viability and germination in intact pollens and pollen tube growth in germinated pollens of five common pomegranate cultivars grown in Croatia and Bosnia and Herzegovina was investigated first time. Pollen viability varied from 36.73% (cv. Konjski Zub) to 51.80% (cv. Barski) in fluorescent diacetat (FDA) test. The average pollen germination percentages were found as the lowest 6.83% in cv. Konjski Zub and the highest 42.51% in cv Glavas. Among the germination media in general 0.2 agar + 10% sucrose+5 ppm H_3BO_3 gave better results to obtain higher pollen germination for all cultivars. In germinated pollens of these five cultivars, significant differences were observed on pollen tube length. Average pollen tube lengths in germinated pollens of five cultivars were measured as minimum 302 μm in cv. Konjski Zub and as maximum 344 μm in cv. Ciparski. The results showed that there were obvious differences in pollen germinability among cultivars growing under the same environmental conditions and these differences must be considered for orchard establishment to obtain higher yield.

Key words: pomegranate, *Punica granatum*, cultivars, pollen germination, pollen tube growth

INTRODUCTION

The pomegranate (*Punica granatum* L.) is one of the oldest known fruits like olive, fig and grape and natively grown from Turkey to Iran and to the Himalayas in northern India. It has also been one of the well known Mediterranean fruits since ancient times

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[Ercisli et al. 2007]. India and Iran both currently dominate world pomegranate production about 750.000 tons production each [Yilmaz 2009]. The other important producer countries are China, Turkey, Egypt, Tunisia, USA, Pakistan, Spain and Morocco. Pomegranate (*Punica granatum* L.) has gained more public attention recently in Balkan countries because of advertisements in media sector on its health components and the number of pomegranate orchard established in Croatia and Bosnia and Herzegovina are increasing parallel to consumers demands as well.

The major pomegranate producer countries have primary commercial pomegranate growing areas and in genaral they have semi-arid mild-temperate to subtropical climate. A humid climate adversely affects the pomegranate plants and fruit cracking is the main problem in these areas. The -13°C is the critical temperature and below this temperature in winter months pomegranate plants severely injured [Onur 2000].

A pomegranate flower has an attractive red calyx and corolla and one flower has about 200–350 anthers. Bisexual flowers contain 400–1000 ovules. Pollen transfer is accomplished by insects and the principal pollinator is the honeybee. To meet export market standards, the fruit has to be of a certain size; this is related to pollination success and consequently to seed number. To ensure a reasonable level of pollination, it is usual to place beehives in orchards during flowering [Derin and Eti 2001].

In vitro pollen viability, germination and pollen tube growth investigations are valuable tools used in identification of the effects of environmental factors and genotypic differences on pollen viability, germination and tube elongation [Gozlekci and Kaynak 2000, Engin and Hepaksoy 2003]. As well known, pollen germination and pollen tube growth are necessary for fertilization, seed and fruit formation in particular for fruit species.

Most plantgrowers use *in vitro* pollen viability, germination and tube growth because of its fast, cheap and simplicity properties in growing programs for identifying favourable cultivars and genotypes which will be used as pollinizer in orchard establishment and breeding objectives [Stosser et al. 1996, Sharafi et al. 2010].

Agar, sucrose and boron are the most widely used medium for determining *in vitro* pollen germination and tube growth [Derin and Eti 2001, Ercisli 2007]. In most fruit species, pollen germination and pollen tube growth are robust under experimentally defined conditions, rendering *in vitro* based studies of relevance to the *in vivo* situation [Cheung 1996, Taylor 1997]. The previous studies on pomegranates showed that pollen viability and germination ratios strongly effected not only by genotypes but also varies depending on methods used and the medium or chemical concentration [Gozlekci and Kaynak 2000, Melgarejo et al. 2000, Derin and Eti 2001, Engin and Hepaksoy 2003]. For this reason, the suitable pollen viability test and germination medium should be determined for each pomegranate cultivars grown in different countries.

In this study, *in vitro* pollen viability, germination capacity and tube growth of pollens belongs to five pomegranate cultivars (Sladun, Barski, Ciparski, Glavas and Konjski Zub) widely grown in both Croatia and Bosnia and Herzegovina were investigated.

MATERIALS AND METHODS

Five pomegranate (*Punica grantum* L.) cultivars (Sladun, Barski, Ciparski, Glavas and Konjski Zub) were used as material. Flower buds in balloon stage of pomegranate cultivars were collected. Plant to plant variation can also be a significant source of variation in pollen viability and germination measurements [Sari-Gorla et al. 1994]. To minimize the effect of this variation without having to perform individual determinations on many plants of single cultivars, pollen from flowers on different plants was taken as a sample in the present study. Fresh pomegranate flowers were collected between 07.30 and 08.30 h, from hermaphodite flowers of five plants per cultivar, and immediately placed in plastic bags and carried to the laboratory. Unopened mature anthers were removed from the flowers and allowed to dry in plastic vials at room temperature. When the anthers dehisced pollen grains were collected in petri dishes and stored in a capped vials at +4°C in refrigerator over crystalline $CaCl_2$ [Ali et al. 1998].

For *in vitro* pollen viability test, fluorescent diacetat (FDA) test procedures were used. In FDA test, 2 mg fluorescent diacetat and 1.71 g sucrose were dissolved in 10 ml distilled water and the pollen were sprinkled. All pollen grains, which fluoresced brightly in a fluorescence microscope were scored as viable. Viability percentages were determined, using four replicates of about 100 grains each [Gozlekci and Kaynak 2000, Ilgin et al. 2007].

The pollen germination experiments were conducted *in vitro* conditions by using agar plate method. Two different media that we named A and B:

– 0.2 agar + 10% sucrose (A)

– 0.2% agar + 10% sucrose + 5 ppm H_3BO_3 (B) were used [Derin and Eti 2001].

The media was placed in petri dishes maintained about 24 and 48 h in 22°C and temperature equilibrated before sprinkling the pollen on the media. Pollen was sprinkled on the media by brush in each petri dish. The whole procedure was completed within 30 min to avoid pollen desiccation. Experimental design was completely randomized design with six replications for each treatments. Pollen germination percentage and pollen tube length were measured under light- microscope (Axioskop 2 plus, Carl Zeiss to increase 100 times) in 10 randomized selected squares after 24 and 48 hours (incubation time). A pollen grain was considered germinated when pollen tube length was at least equal to or greater than the grain diameter. Measurements of pollen tube length were recorded directly by AxioVision 4 Software system for analysis and processing photos. Data were analyzed using SAS software and comparison of means was carried out with Duncan's multiple range tests.

RESULTS AND DISCUSSION

Results of pollen viability FDA test showed significant differences among cultivars at $p < 0.01$ level (Table 1).

The viable pollen ratio in FDA test were higher in cv. Glavas as 52.15% and the lowest in cv. Konjski Zub (36.73%), respectively. Gozlekci and Kaynak [2000] reported that pollen viability varied between 40–60% in pomegranate cultivars by using FDA

test. Ali et al. [1998] found 60–97% pollen viability by using acetocarmine dye method eleven pomegranate cultivars in Saudi Arabia. Derin and Eti [2001] studied on flower biology of two well known pomegranate cultivars in Turkey (cvs Hicaznar and 33 N 26) and they found that the highest pollen viability was obtained from cv. Hicaz as 82.45% viability in FDA test.

Table 1. Pollen viability and germination ratio (%) in pomegranate cultivars
Tabela 1. Żywotność pyłku i współczynnik kiełkowania (%) u odmian granatu

Cultivar Odmiana	Average pollen viability Średnia żywotność pyłku (%) (Test FDA)	Medium Pożywka	Incubation time Czas inkubacji (h)	Pollen germination ratio Współczynnik kiełkowania pyłku (%)	Average pollen germination ratio Średni współczynnik kiełkowania pyłku (%)
Sladun	42.46b	A	24	32.06	31.20b
			48	20.88	
		B	24	35.63	
			48	36.22	
Barski	51.80a	A	24	33.75	24.75c
			48	15.25	
		B	24	29.61	
			48	19.15	
Ciparski	41.10b	A	24	30.30	28.84bc
			48	16.63	
		B	24	35.61	
			48	32.83	
Glavas	52.15a	A	24	38.80	42.51a
			48	29.14	
		B	24	53.11	
			48	49.00	
Konjski zub	36.73c	A	24	5.90	6.83d
			48	3.60	
		B	24	12.38	
			48	14.00	

Medium: A (0.2% agar + 10% sucrose); B (0.2% agar + 10% sucrose + 5 ppm H_3BO_3) – Pożywka A (0,2% agar + 10% sacharoza); B (0,2% agar + 10% sacharoza + 5 ppm H_3BO_3)
*Different letters indicate the statistical differences within the same column among cultivars at 1% level – Różne litery oznaczają różnice statystyczne pomiędzy odmianami w tej samej kolumnie na poziomie 1%

The results of the effect of different media and incubation times on pollen germination percentage and pollen tube length of five pomegranate cultivars are shown in Tables 1 and 3, respectively. There were significant differences among cultivars both for germination percentage and pollen tube length at $p < 0.01$ level (tab. 1 and 3). The overall effects of media on pollen germination and pollen tube length were found statistically important ($p < 0.01$). In addition significant differences among incubation times on pollen germination was observed (tab. 2) but incubation times did not effected pollen tube length (tab. 4). The all interactions related to pollen viability, germination and tube length were found insignificant.

The average highest pollen germination percentages were obtained from cv. Glavas as 42.51% followed by cv. Sladun (31.20%) and cv. Ciparski (28.84%), respectively.

The cultivar Konjski zub overall experiment showed the lowest pollen germination percentage (6.83%) (tab. 1). For the germination media tested considering the average values of five cultivars, 0.2% agar + 10% sucrose + 5 ppm H_3BO_3 were found to be more suitable combinations for all searched pomegranate cultivars to obtain higher pollen germination percentage (tab. 2). The average germination percentage was 31.75% in this medium compared to 0.2 agar + 10% sucrose without H_3BO_3 (22.63%) (tab. 2). This results are highlighting the importance of H_3BO_3 in germination medium. Melgarejo et al. [2000] conclude that the optimum sucrose concentration were between 10 and 20% to obtain higher pollen germination in pomegranate cultivars and they also stated that the sucrose levels below 10% producing low levels of pollen germination. The authors did not found statistical significant differences between sex factor (A and B type flowers) and pollen germination percentage in pomegranate. Engin and Hepaksoy [2003] used flowers of eleven pomegranate cultivars in pollen germination study in western Turkey and they found that pollen germination ratio is higher in media containing 15 and 20% sucrose. They observed that average pollen germination of Izmir 2 (39.53%) and Izmir 1261 (34.13%) cultivars were higher than others. Ali et al. [1998] found between 68 and 92% pollen germination ratio by using hanging drop method among eleven pomegranate cultivars in Saudi Arabia and Gozlekci and Kaynak [2000] reported pollen germination between 27.43 to 68.25% in pomegranate cultivars by using 0.2% agar + 10% sucrose + 5 ppm H_3BO_3. Our results are comparable Engin and Hepaksoy [2003] and Gozlekci and Kaynak [2000], respectively. Gozlekci and Kaynak [2000] also stated that variable external factors such as humidity, temperature and ingredients of the substrate used for germination may have an effect on pollen germination. In our study 24 hour incubation time gave higher (30.72%) pollen germination percentage than 48 hour incubation period (23.67%) (tab. 2).

Table 2. The effects of time and medium on pollen germination ratio (%) in 5 pomegranate cultivars

Tabela 2. Wpływ czasu i pożywki na współczynnik kiełkowania pyłku (%) u 5 odmian granatu

Time Czas (h)	Pollen germination ratio Współczynnik kiełkowania pyłku (%)	Medium Pożywka	Pollen germination ratio Współczynnik kiełkowania pyłku (%)
24	30.72a	A	22.63b
48	23.67b	B	31.75a

Medium: A (0.2% agar + 10% sucrose); B (0.2% agar + 10% sucrose + 5 ppm H_3BO_3) – Pożywka A (0,2% agar + 10% sacharoza); B (0,2% agar + 10% sacharoza+5 ppm H_3BO_3)
NS: Non significant – NS: nieistotne
*see: Table 1 – Patrz: tabela 1

The cultivars placed same order both in pollen viability and germination test results suggesting there were strong relationships between pollen viability and pollen germination in pomegranate cultivars. This strong correlation has been reported before in fig [Ilgin et al. 2007] and rose hip [Ercisli 2007].

We are also found that low boric acid concentration was more effective to stimulate pollen germination and pollen tube growth in pomegranates. Sharafi et al. [2010] reported that higher boric acid concentrations inhibited pollen germination and tube elongation in rose hip. In this study, for all cultivars pollen viability was found higher than pollen germination. This result was in accordance with previous studies [Ercisli 2007, Ilgin et al. 2007, Beyhan et al. 2009]. Various germination test and media as well as culture conditions may affect the germination results of a given cultivar. Although intact (viable) pollen might be expected to have a good germination capacity, lower germination percentages are often obtained, possibly due to insufficient *in vitro* procedures. In our study, viability tests indicated the existence of moderate percentage of viable pollen in pomegranates grown in Croatia and Bosnia Herzegovina.

Pomegranate cultivars differed significantly in pollen tube length at different media and incubation times (tab. 3 and 4). Pollen tube length ranged from 302 μm for cv. Konjski zub and reached the maximum 344 μm in cv Ciparski (tab. 3). The overall highest pollen tube length were observed in 0.2% agar + 10% sucrose + 5 ppm H_3Bo_3 medium as 384 μm compared to 0.2 agar + 10% sucrose without H_3Bo_3 (256 μm) according to average of cultivars.

Table 3. Pollen tube length (μm) in pomegranate cultivars
Tabela 3. Długość łagiewki pyłkowej (w μm)

Cultivar Odmiana	Medium Pożywka	Incubation time Czas inkubacji (h)	Pollen tube length Długość łagiewki pyłkowej (μm)	Mean pollen tube length Średnia długość łagiewki pyłkowej (μm)
Sladun	A	24	316	303c
		48	210	
	B	24	338	
		48	348	
Barski	A	24	372	322b
		48	199	
	B	24	348	
		48	367	
Ciparski	A	24	305	344a
		48	227	
	B	24	413	
		48	432	
Glavas	A	24	298	330ab
		48	182	
	B	24	433	
		48	406	
Konjski zub	A	24	224	302c
		48	228	
	B	24	361	
		48	396	

Medium: A (0.2% agar + 10% sucrose); B (0.2% agar + 10% sucrose + 5 ppm H_3BO_3) – Pożywka A (0,2% agar + 10% sacharoza); B (0,2% agar + 10% sacharoza +5 ppm H_3BO_3)
* See: Table 1 – Patrz: tabela 1

Table 4. The effects of time and medium on pollen tube length in 5 pomegranate cultivars
Tabela 4. Wpływ czasu i pożywki na długość łagiewki pyłkowej u 5 odmian granatu

Time Czas (h)	Pollen tube length Długość łagiewki pyłkowej (µm)	Medium Pożywka	Pollen tube length Długość łagiewki pyłkowej (µm)
24	311^{NS}	A	256b
48	300	B	384a

Medium: A (0.2% agar + 10% sucrose); B (0.2% agar + 10% sucrose + 5 ppm H_3BO_3) – Pożywka A (0,2% agar + 10% sacharoza); B (0,2% agar + 10% sacharoza + 5 ppm H_3BO_3)
NS: Non significant – NS: nieistotne
* See: Table 1 – Patrz: tabela 1

The previous results from *in vitro* studies with different fruit species showed that genotypes varied in response to different media for high pollen germination percentage and maximum pollen tube length [Ali et al. 1998, Melgarejo et al. 2000, Ilgin et al. 2007, Beyhan and Serdar 2009, Sharafi et al. 2010]. It seems that 0.2% agar + 10% sucrose + 5 ppm H_3Bo_3 medium which suggested for pomegranates before [Gozlekci and Kaynak 2000] is better for pomegranate cultivars to obtain higher pollen germination. Previous studies clearly indicated that the presence of boric acid in germination media had positive effect on pollen germination and pollen tube length in plant species [Melgarejo et al. 2000, Wang et al. 2003, Tosun and Koyuncu 2007, Sharafi et al. 2010]. Wang et al. [2003] reported that callose accumulated in the tip-regions of pollen tubes cultured in boron-deficient medium, but not in standard medium in *Picea meyeri*. They indicated that acidic pectin preferentially accumulated in the tip regions of pollen tubes cultured in boron-deficient medium, whereas acidic pectin was weakly distributed along the entire lengths of pollen tubes cultured in standard medium. They used FTIR spectra and they found slight increases in contents of phenolics and carboxylic acids and a substantial decrease in the content of saturated esters in boron-deficient pollen tubes compared with normal pollen tubes. The FTIR spectra confirmed that boron deficiency enhanced acidic pectin accumulation in pollen tubes, which may be associated with the increased content of carboxylic acid and finally they conclude that boron has a regulatory role in pollen germination and pollen tube growth.

To determine the pollen viability, pollen germination and tube length is important in particular orchard establishment in pomegranates to obtain higher yield by selecting suitable pollinizers. Determining the best viable test and germination media are also important for pomegranates for future breeding efforts particularly to determine parents for crossing. To investigate pollination potential, estimates should be made of pollen quantity and viability, as well as of pollen germination capability for breeding and growing programs [Ercisli 2007].

CONCLUSION

In determining the pollen quality, viability tests are often considered to be faster and easier methods than the germination tests, since the effects of external factors such as temperature, humidity, and germinating media are minimized. The results of our study strongly supported this approach. Based on the results, it is concluded that higher pollen germination percentages and longer pollen tubes were obtained in 0.2% agar + 10% sucrose + 5 ppm H_3BO_3 media. Significant cultivar differences for pollen viability, germination and pollen tube growth were also observed in the present study. The cultivars that had the highest pollen viability also had high germinated pollen viability. These cultivars can be important for the future pomegranate breeding, as well as growing programs in Croatia and Bosnia and Herzegovina. The low pollen germinated cultivar cv. Konjski zub must be mixed with other cultivars in new established orchards to obtain enough crop.

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ŻYWOTNOŚĆ PYŁKU *in vitro,* KIEŁKOWANIE I WZROST ŁAGIEWKI PYŁKOWEJ U NIEKTÓRYCH ODMIAIN GRANATU (*Punica granatum* L.) Z CHORWACJI ORAZ BOŚNI I HERCEGOWINY

Streszczenie. W niniejszej pracy po raz pierwszy zbadano żywotność pyłku *in vitro* oraz kiełkowanie w nieuszkodzonych pyłkach oraz wzrost łagiewki pyłkowej w kiełkujących pyłkach pięciu popularnych odmianach granatu hodowanych w Chorwacji, a także Bośni i Hercegowinie. Żywotność pyłku wahała się od 36,73% (odm. Konjski Zub) do 51,80% (odm. Barski) w teście dwuoctanu fluorescencyjnego (FDA). Średni procent kiełkowania pyłku kształtowało się na najniższym poziomie 6,83% w odmianie Konjski Zub, a na najwyższym –42,51% w odmianie Glavas. Spośród pożywek do kiełkowania generalnie 0,2 agar + 10% sacharoza + 5 ppm H_3BO_3 dawały lepsze wyniki w uzyskiwaniu wyższego poziomu kiełkowania pyłku dla wszystkich odmian. W pyłkach kiełkujących tychże pięciu odmian znaczące różnice zaobserwowano w długości łagiewki pyłkowej. Zmierzono średnie długości łagiewki pyłkowej u pięciu odmian, a długość minimalna wynosiła 302 µm w odmianie Konjski Zub, a maksymalna 344 µm w odmianie Ciparski. Wyniki wskazywały, że istniały oczywiste różnice w zdolności kiełkowania pyłku u odmian rosnących w tych samych warunkach środowiskowych i różnice te należy brać pod uwagę przy zakładaniu sadu, aby uzyskać wyższy plon.

Słowa kluczowe: granat, *Punica granatum*, odmiany, kiełkowanie pyłku, wzrost łagiewki pyłkowej

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