HORSE CHESTNUT POLLEN QUALITY

Dušica ĆALIĆ, Ljiljana RADOJEVIĆ

Department of Plant Physiology, Institute for Biological Research “Siniša Stanković”,
University of Belgrade, Belgrade, Serbia


Pollen quality of horse chestnut, expressed as pollen productivity, viability and germination was studied. Anthers of horse chestnut genotypes had pollen production from 3.66 to 5.06 x 10³ pollen grains per anther, depending of genotype. Also, pollen of horse chestnut Ah1-Ah4 genotypes showed different viability (from 56 to 68%), after staining with fluorescein diacetate. Pollen germination of Ah1-Ah4 genotypes varied from 50-66% on basic medium. Inclusion of polyethylene glycol-PEG from 10%, 15% and 20% v/w increased pollen germination. The best results were achieved on medium with the largest PEG concentration. On these medium 76-91% pollen grains were germinated, depending of genotype. The best pollen quality, for all tested parameters, had genotype Ah2. Knowledge about morphology, production, viability, in vitro germination, tube growth as well as pollen: ovule ratio can be of great importance for future pollen biology studies.

Key words: Aesculus, pollen morphology, pollen:ovule ratio, pollen tube growth, pollen viability

INTRODUCTION

European horse chestnut (Aesculus hippocastanum L.; Sapindaceae: Hippocastanaceae) is an attractive ornamental plant. Horse chestnut is a relict and endemic species of the Balkan Peninsula (Stevanović and Radojević, 1993; Prada et al., 2011) and important medical plant (Calić et al., 2003; Calić-Dragosavac et al., 2010).

Corresponding author: Dušica Ćalić, Department of Plant Physiology, Institute for Biological Research “Siniša Stanković”, University of Belgrade, Belgrade, Serbia, Tel.: +381-11-2078-366; fax: +381-11-2761 433, E-mail address: calic@ibiss.bg.ac.rs
Seeds of horse chestnut are the main source of escine, which is used in the treatment of peripheral blood vessels. Pollen quality which includes pollen morphology, pollen production, pollen: ovule ratio, pollen viability, pollen germination and tube growth, is an important component of fertilization success in seed-producing plants.

Pollen production has been an important process in the field of plant reproductive biology. For example, the amounts of pollen produced by a plant and anther position (Harder and Barrett, 1993) are a critical component of male reproductive function (Kearns and Inouye, 1993). Counting pollen can also be an important part of plant–pollinator studies, such as quantifying the amount of pollen that is removed and carried by pollinators (Thomson and Goodell, 2001).

Many studies have indicated that viability, germination and tube growth of pollen grains varied significantly according to species and cultivar (Hedly et al., 2004; Du et al., 2006; Atlagić et al., 2009; Sharafi, 2011; Souza et al., 2015). Pollen performance is clearly affected by the genotype (Vuletin Selek et al., 2013) which is why pollen development and morphology are often used by taxonomists and paleobotanists to clarify the classification and identity of plant species (Mert, 2009).

A variety of factors influencing pollen performance such as temperature (Koubouris et al., 2009; Acar and Kanani, 2010; Beck-Pay, 2012; Cerović et al., 2014), sucrose (Alcaraz et al., 2011; Devrnja et al., 2012), polyethylene glycol-PEG (Sakhankho and Rajasekaran, 2010; Čalić et al., 2013) were tested. Furthermore, the ratios of pollen grains to number of ovules (P: O ratios) have been shown to correlate with the mating system of a plant. Cruden (1977), Muchhala et al. (2010) and Pang and Saunders (2015) were demonstrated that in general, xenogamous species had higher P: O ratios than predominantly selfing species and conclude that xenogamous species had higher P: O ratios while autogamous species had lower P: O ratios. This pollination efficiency hypothesis is based on the argument that maximum seed set in xenogamy plants would require more pollens grains as result of inefficient pollen transfer.

Knowledge about morphology, production, viability, in vitro germination, tube growth as well as pollen: ovule ratio can be of the most importance for understanding not only the basic characteristics of horse chestnut pollen but also its pollination biology.

The aim of the present study was to determine and compare pollen production, pollen: ovule ratio, pollen viability, pollen germination and tube growth capacity of A. hippocastanum genotypes.

**MATERIALS AND METHODS**

**Plant materials**

The study was conducted on flowers from the lower branches of four genotypes of A. hippocastanum (voucher N0, Ah1; Ah2, Ah3, Ah4). The samples were collected from 125-year old trees growing in the Botanical Garden “Jevremovac” of the Belgrade University. For experiments, flowers at blooming phase were used.

**Scanning electron microscopy**

Anther and pollen of Ah1-Ah4 genotype samples were collected from fresh flowers, and processed for scanning microscopy (SEM) without the usual fixation and dehydration procedures. Anthers and pollen grains were placed directly on the stubs, and were covered with a thin layer of gold (ion sputtering coating) in a BALETECSCD 005 Sputtering Device, imaging at 15 kV, using...
a JSM-6390 LV (JEOL, Tokyo, Japan) scanning electron microscope. Scanning electron micrograph images were taken on magnification from 45× to 13000× (Fig. 2a-e). The measurements were made in micrometer (μm).

**Pollen count and pollen : ovule ratio**

Average number of pollen grains per anther, flower and inflorescence was counted on 180 randomly chosen flowers and 30 inflorescences. Six flowers per inflorescence were analyzed for each genotype.

The drying and dehiscence of anthers in vial were obtained first at room temperature, then in a stove at 50°C for 6 hours. Too many pollen grains stuck to the endotheciums, so that it was necessary to free most of the grains by triturating carefully the anthers with a glass rod. Two ml of 0.002 % aqueous solution of a detergent (Tween 20, Sigma) were poured in each vial and a uniform suspension of grains was obtained after shaking.

The chambers of the "Bürker" haemocytometer were filled with drops of the suspension with micropipette.

Also, for content screening of anthers, anthers were cut longitudinally and stained with 1% carmine solution prepared in 45% acetic acid (Fig. 1d). Acetocarmine treated anthers were observed under DMRB microscope from Leica (Wetzlar, Germany).

The counts of grains in each of the 9 small chambers were averaged and multiplied x 100. This procedure was repeated six times per vial.

The lower portion of the corolla tube was cut off and split open with a scalpel to reveal the ovary. The ovary was then placed under a light magnifier, and the ovules were carefully removed with two dissecting needles. Subsequently, ovules were spread out and counted. For each flower, the pollen : ovule ratio (P: O) was determined by dividing the number of pollen grains per anther by the number of ovules. For each genotype, 180 randomly chosen flowers were used.

**Pollen viability in vitro**

To study pollen viability *in vitro*, anthers were sampled at late balloon stage. Pollen viability was precisely detected with fluorescein diacetate following HESLOP-HARRISON and HESLOP-HARRISON (1970). A fresh fluorescein diacetate (FDA) in acetone at a concentration of 2 mg mL⁻¹ was added diluted by 0.5 M sucrose solution (1:1) up to saturation. One droplet was deposited on each pollen sample. Slides were mounted with a coverslip and observations were made with a Zeiss Axiovert fluorescent microscope equipped for reflected-light fluorescence with a UV mercury lamp. Digital images of the microscope view were obtained with a digital camera (AxioCamMRC3, Carl Zeiss), after 24- and 48 hours of FDA staining.

In addition viable, semi-viable and dead pollen numbers and their percentages were determined. Viable pollen was dyed in green, semi viable pollen dyed in light green and dead pollen was not dyed.

**In vitro pollen germination and tube growth**

For the pollen germination test, the drop technique was used (ČALIĆ et al., 2013). A "basic" liquid medium containing 1.2 M sucrose, 0.3 g L⁻¹ calcium nitrate [Ca(NO₃)₂], 0.10 g L⁻¹ boric acid (H₃BO₃), 0.1g L⁻¹ potassium nitrate (KNO₃), and 0.2 g L⁻¹ magnesium sulfate (MgSO₄ 7H₂O) was used. The effect of polyethylene glycol (PEG) concentrations (10, 15, and 20%, w/v) on pollen germination and tube growth was evaluated. PEG-free medium was used as a
control. For the germination test with the PEG-based media, both pollen germination and pollen tube growth were recorded. Counts of germinated pollen grains were made under a light microscope (DMRB microscope from Leica Wetzlar, Germany) after 24 h.

Mean pollen tube length was calculated as the average length of 200 pollen tubes measured from each slides after 24 h. Three replicates (slides) were used for each genotype. A pollen grain was considered to have germinated when pollen tube length equaled or exceeded the grain diameter. Germination percentage was determined by dividing the number of germinated pollen grains by the total number of pollen grains per field of view and multiplying by 100. To measure pollen tube growth, samples were prepared as described above, and the slides were observed under the Leica (Wetzlar, Germany) light microscope equipped with a camera.

Statistical analysis

The measurements of the diameter, viability, nuclei status and germination were taken on 600 pollen grains for each genotype. The means were separated using FISHER’s LSD post hoc test for $P \leq 0.05$. Statistical analysis was performed using OriginPro 8 (OriginLab Corporation) and Statistica 8 (StatSoft, Inc.).

RESULTS

Characteristics of inflorescence

The flowers of horse chestnut were arranged in pyramidal inflorescence. This means that multiple flowers were grouped together into clusters. The flowers of A. hippocastanum had white spot (Fig. 1a). These flowers were produced in spring in erect inflorescences. All genotypes of A. hippocastanum had inflorescences up to 30 cm length.

Figure 1. Inflorescence, flower, anthers and pollen morphology of A. hippocastanum photographed under a binocular magnifier and light microscope. (a) Inflorescence morphology; (b) Detail flower morphology photographed under a binocular magnifier; (c) Anther morphology; (d) Lengthwise dissection of acetocarmine stained anther. Darker (viable) colored and lighter (nonviable, arrow) pollen grains. Anther hairs (arrow). Scale bars for (a)= 3 cm, (b)= 2 cm, (c)= 3 mm, (d)= 250 μm
**Anther and pollen morphology**

Anther morphology of horse chestnut is shown in Fig. 1b, c, 2a. Lengthwise dissection of acetocarmine stained anthers showed that pollen grains were densely packed into the anther (Fig. 1d).

Anther hairs were noticed on anthers of all *A. hypopcastanum* genotypes (Fig. 1b-d; 2a-c). The pollen of all genotypes was tricolporate while the shape of pollen was circular in the polar view and elliptical in the equatorial view (Fig. 2c, d). The number of colpi (furrows) was three while the spines were absent. Exine ornamentation of mature pollen grains was striate (Fig. 2d, e). Pore of exine, diameter from 0.05 to 0.15 μm were roundish or oval. Four perforations (Fig. 2e) were noticed on 1 μm² of exine. Pollen grains within a species, not depending on the genotype, have the same morphology.

![Figure 1. Scanning electron micrographs of anther and pollen morphology of *A. hypopcastanum*. (a) Anther morphology; (b) Detail of anther surface; (c) Pollen grains in different views; (d) Equatorial view of pollen grain with a specific exine ornamentation; (e) Striate detail of exine with pores. Anther hairs and perforation (arrow)](image)

**Pollen production and pollen: ovule ratio**

Each genotype of horse chestnut was significantly different compared by pollen number per anther and pollen number per flower. Genotypes were not significantly different compared by the number of flowers per inflorescence. Genotypes Ah1 and Ah2 had larger number of flowers per inflorescence, but lower production of pollen grains per anther, flower and inflorescence (Table 1). The pollen production in anthers of *A. hypopcastanum* genotypes varied from 3.66-5.06 x 10³, depending of genotype (Table 1).
P: O ratio varied from 610 to 844, while the best performing was genotype Ah2. However, no significant differences in ovule number and number of anthers per flower were found between analysed genotypes (Table 1).

<table>
<thead>
<tr>
<th>Species</th>
<th>Gen.</th>
<th>F/I</th>
<th>O/F</th>
<th>PG/A</th>
<th>PG/F</th>
<th>A/F</th>
<th>P:O</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. hippocastanum</td>
<td>Ah1</td>
<td>205 a</td>
<td>6.0 a</td>
<td>4.38 x 10^3 b</td>
<td>3.07 x 10^4 ab</td>
<td>6.78 a</td>
<td>830 a</td>
</tr>
<tr>
<td></td>
<td>Ah2</td>
<td>208 a</td>
<td>6.0 a</td>
<td>5.06 x 10^3 a</td>
<td>3.48 x 10^4 a</td>
<td>6.88 a</td>
<td>844 a</td>
</tr>
<tr>
<td></td>
<td>Ah3</td>
<td>199 a</td>
<td>6.0 a</td>
<td>3.66 x 10^3 c</td>
<td>2.50 x 10^4 b</td>
<td>6.82 a</td>
<td>610 c</td>
</tr>
<tr>
<td></td>
<td>Ah4</td>
<td>197 a</td>
<td>6.0 a</td>
<td>4.16 x 10^3 b</td>
<td>2.87 x 10^4 ab</td>
<td>6.91 a</td>
<td>693 b</td>
</tr>
</tbody>
</table>

*Same letters show no significantly difference among species and genotypes of each column (p<0.05)*

**Pollen viability in vitro**

We noticed viable, semi-viable and dead FDA treated pollen grains in A. hippocastanum after 24- and 48 hours. After 24 h, depending of genotypes, pollen viability varied from 56 to 68%. The best results showed Ah2 genotype (Table 2). However, differences between 24 and 48 hour pollen viability rates were statistically not significant. Therefore, 24 hours may be enough for detection of pollen viability.

<table>
<thead>
<tr>
<th>Species</th>
<th>Genotype</th>
<th>Viable</th>
<th>Semiviable</th>
<th>Dead</th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>24h</td>
<td>48h</td>
<td>24h</td>
</tr>
<tr>
<td>A. hippocastanum</td>
<td>Ah1</td>
<td>62 ab</td>
<td>63 ab</td>
<td>6 ab</td>
</tr>
<tr>
<td></td>
<td>Ah2</td>
<td>68 a</td>
<td>68 a</td>
<td>7 a</td>
</tr>
<tr>
<td></td>
<td>Ah3</td>
<td>56 b</td>
<td>57 b</td>
<td>4 b</td>
</tr>
<tr>
<td></td>
<td>Ah4</td>
<td>59 b</td>
<td>60 b</td>
<td>2 b</td>
</tr>
</tbody>
</table>

*Same letters show no significantly difference among species and genotypes of each column (p<0.05)*

**In vitro pollen germination and tube growth**

Pollen germination varied from 50-66% on basic medium, depending of genotype (Table 3). Inclusion of polyethylene glycol-PEG from 10%, 15% and 20% v/w increased pollen germination. The best results were achieved on medium with the largest concentration of about 20% PEG. On this medium 76-91% pollen grains of A. hippocastanum were germinated, depending of genotype. The best germination had genotype Ah2.

PEG had the same impact on pollen tubes elongation as well as on pollen germination in all genotypes (Table 4, Fig. 3). So, the highest pollen tube length (1188 μm) had Ah2 genotype on medium with 20% PEG.
Table 3. Germination of *A. hippocastanum* pollen on different PEG concentration after 24h

<table>
<thead>
<tr>
<th>Species</th>
<th>Genotype</th>
<th>Germination (%)</th>
<th>PEG concentration (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0</td>
<td>10</td>
</tr>
<tr>
<td><em>A. hippocastanum</em></td>
<td>Ah1</td>
<td>59 ab</td>
<td>62 b</td>
</tr>
<tr>
<td></td>
<td>Ah2</td>
<td>66 a</td>
<td>69 a</td>
</tr>
<tr>
<td></td>
<td>Ah3</td>
<td>50 c</td>
<td>55 c</td>
</tr>
<tr>
<td></td>
<td>Ah4</td>
<td>54 b</td>
<td>60 b</td>
</tr>
</tbody>
</table>

*Same letters show no significantly difference among species and genotypes of each column (p<0.05)*

Table 4. Tube growth of *A. hippocastanum* pollen on different PEG concentration after 24h

<table>
<thead>
<tr>
<th>Species</th>
<th>Genotype</th>
<th>Tube growth (μm)</th>
<th>PEG concentration (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0</td>
<td>10</td>
</tr>
<tr>
<td><em>A. hippocastanum</em></td>
<td>Ah1</td>
<td>114 b</td>
<td>208 b</td>
</tr>
<tr>
<td></td>
<td>Ah2</td>
<td>178 a</td>
<td>313 a</td>
</tr>
<tr>
<td></td>
<td>Ah3</td>
<td>75 d</td>
<td>110 c</td>
</tr>
<tr>
<td></td>
<td>Ah4</td>
<td>91 c</td>
<td>179 bc</td>
</tr>
</tbody>
</table>

*Different letters show significantly difference among species and genotypes of each column (p<0.05)*

Figure 3. Morphology of *A. hippocastanum* germinating pollen on medium with 10% (a), 15% (b) and 20% (c) sucrose. Scale bars= 200 μm

**DISCUSSION**

SEM images showed that these pollen grains are tricolporate with a well-defined exine with specific architecture and ornamentation. In general, pollen grains of terrestrial taxa have well-developed and defined exine (MARTINSSON, 1993; OSBORN and PHILBRICK, 1994; COOPER et al., 2000). The role of the exine is to protect the male spore and gametophyte from desiccation and other dangers of sub-aerial dispersal.

Our measurement of equatorial axis, the polar axis and diameter of the pollen grains of *A. hippocastanum* genotypes are similar with previously reported results for the genus *Aesculus* (HEATH, 1984; POZHDIAEV, 1995; KONYAR, 2012).
Such morphological characteristics of pollen can have implications for pollen germination, pollen viability as well as pollination mechanisms. TANAKA et al. (2004) concluded that the selective pressures acting on the pollination mechanisms have resulted in various exine sculptures that are adapted to the different pollination mechanisms in entomophilous and anemophilous plants.

Genotypes were significantly different in pollen production per anther, unlike for flower and inflorescence, as they contained the same anther number per flower and similar number of flowers per inflorescence. These results are supported by previously published findings on horse chestnut (SZKLANOWSKA and STRZALKOWSKA, 2000; WERYSZKO-CHMIELEWSKA et al., 2012) and walnut (SÜTYEMEZ, 2007). Pollen viability and germination varied between horse chestnut genotypes, as was found for oak, almond and peach genotypes (GÓMEZ-CASERO et al., 2004; IMANI et al., 2011).

There is some data about of acidity and detergent effects on in vitro horse chestnut pollen germination and tube growth (PAOLETTI, 1992). In our research, the use of PEG is proved very effective in stimulating of horse chestnut pollen germination. However, all A. hippocastanum genotypes showed significant differences in pollen germination and pollen tube elongation. Indeed, highest PEG (20 %) concentration ensured almost complete pollen germination (91 %) of Ah2 horse chestnut genotype. Previous studies showed that PEG functions as an osmoticum and improves in vitro pollen germination and tube growth by preventing tube bursting (SAKHANOKHO and RAJASEKARAN, 2010; ČALIĆ et al., 2013).

The P: O ratio has traditionally been used as a rough estimator of plant breeding systems. It has been shown that plant breeding systems are associated with particular floral traits. In this study, we determined the P: O in horse chestnut and explored relationships between P: O and pollen presentation and pollination mechanisms. This species showed high P: O ratios. Horse chestnut, on the basis of P: O ratio may be classified as facultative xenogamous according to CRUDEN (1977). It is common for xenogamous (outcrossed) hermaphroditic flowering plants to produce more flowers and ovules than fruits and seeds (CRUDEN, 1977, 2000; BAWA and WEBB, 1984; MUCHHALAE et al., 2010; PANG and SAUNDERS, 2015; LI et al., 2016). Accordingly, horse chestnut produced many more flowers than fruits. Our results demonstrate that P: O variability is determined by pollination mechanism in horse chestnut species.

The presented horse chestnut pollen study could be a test model for pollen quality as essential component for successful fertilization in seed-producing plants.

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REFERENCES


KVALITET POLENA DVLJEG KESTENA
Dušica ĆALIĆ, Ljiljana RADOJEVIĆ
Odeljenje za Fiziologiju biljaka, Institut za Biološka Istraživanja “Siniša Stanković“, Univerzitet u Beogradu, Beograd, Srbija

Izvod
Proučavan je kvalitet polena divljeg kestena, izražen kao produktivnost, vijabilnost i klijavost. Prašnici divljeg kestena produkuju od 3.66 do 5.06 x 10^3 polena po anteri, u zavisnosti od genotipa. Polen genotipova Ah1-Ah4 divljeg kestena pokazuje različitu vijabilnost (od 56 do 68%), nakon bojenja fluorescein diacetatom. Klijanje polena Ah1-Ah4 genotipova varira od 50 do 66% na osnovnoj hranljivoj podlozi. Međutim, uključivanjem polietilenglikola-PEG (10%, 15% i 20%) povećava se klijavost polena. Najbolji rezultati postignuti su na hranljivoj podlozi s najvećom koncentracijom PEG-a. Na ovoj podlozi klijalo je 76-91% polena divljeg kestena, u zavisno od genotipa. Najbolji kvalitet polena, po svim testiranim parametrima, ima genotip Ah2. Poznavanje morfologije polena, njegove produkcije, vijabilnosti, klijanja in vitro, rasta polenove cevi, kao i odnosa polen: neoplođeno jaje, može biti od velike važnosti za buduće studije biologije polena.

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