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Pollen viability in *Quercus robur* l.

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Abstract: The variability of viability (germination rate and the length of pollen tubes) of fresh pedunculate oak (*Quercus robur* L.) pollen grains was studied *in vitro* on a medium containing 15% sucrose. Spatial variability was studied by sampling fresh pollen grains from a total of thirteen trees at four different sites in the area of Belgrade (Košutnjak, Banovo Brdo, Ada Ciganlija and Bojčin Forest) in a single year (2011). In order to assess temporal variability and determine the effects of climate change on a small time scale, we studied the viability of the pollen grains collected from one tree at the Banovo Brdo site in six different years (2004, 2005, 2006, 2007, 2011 and 2012). Interindividual variability was tested on the pollen grains sampled from eight trees at Ada Ciganlija in 2004. The percentage values of the pollen grain germination rate and the pollen tube length showed no statistically significant differences between the sites. However, the studied characteristics of the pollen grain viability (germination rate and pollen tube length) showed statistically significant differences in both temporal (between the pollen collection years) and interindividual variability. This type of research makes a valuable contribution to pedunculate oak breeding programs through the identification of trees with stable production and a good quality of pollen. Furthermore, it can be important in defining the patterns of spatial, temporal and individual variability of pollen grain viability under the influence of climate factors, which are showing compelling changing trends from year to year.

Key words: pollen; pedunculate oak; pollen tube length; germination rate

INTRODUCTION

Oaks (*Quercus*) make up the most numerous genus of the *Fagaceae* family. They have a wide range of distribution in the temperate zone of the northern hemisphere. Pedunculate oak (*Quercus robur* L.) is the most common oak species in Europe. Global climate change and the resulting changes in site conditions have contributed to a significant reduction in the surface area covered by pedunculate oak [1-3].

Pedunculate oak is native to Serbia and together with beech and sessile oak it is one of the most common and most valued forest tree species [4]. Its wide range of distribution contributes to its high individual morphological, anatomical, phenological and physiological variability [5].

Pedunculate oak is a monoecious anemophilous species. Male and female flowers are in separate clusters, with male flowers (catkins) developing on the previous year's shoots and female flowers on the current year's shoots. Pedunculate oaks flower irregularly and produce different quantities of pollen, which makes their natural regeneration more difficult. They flower abundantly every 2-3 years, usually in spring (April/May), or sometimes later in summer (July/August). Summer pollen is often dysfunctional and it has different morphological and physiological traits from the pollen produced in spring [5]. Morphological pollen grain traits are largely genetically determined, while its physiological characteristics depend not only on genetic, but also on environmental factors [6-8]. Pollen grain quality (high germination rate and the length of pollen tubes, i.e. long pollen tubes) affects fertilization, fruiting, seed quality and ultimately the survival and recovery of a plant community. Furthermore, intraspecies variability can be used to identify pollen donor trees, i.e. trees that produce good quality pollen [9], which is very important from the aspect of tree breeding.

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Oak pollen has been the subject of interest in studies dealing with morphology as part of systematics and hybridization [10-16] or paleobotany and plant migration and evolution [17-22], aerobiology and pollen production [23-27]. Most of these studies deal with pollen at the species level. However, the number of studies dealing with individual variability of oak pollen and specific germination conditions is significantly smaller [28-30].

The aim of this study was to examine spatial, temporal, and individual variability of pedunculate oak pollen grain viability. We attempted to find answers to the following questions, which should help geneticists and breeders in breeding programs related to this species: i) Is there spatial variability in the viability of the pollen grains collected in the same year? ii) Can climate factors observed on a small time scale of nine years affect the variability of pollen grain germination and pollen tube length? iii) Do the studied characteristics of pedunculate oak pollen show interindividual variability in the same environmental conditions?

MATERIALS AND METHODS

Study sites and sample collection

Three studies were undertaken to examine the viability (germination rate and energy of germination) of fresh pedunculate oak pollen collected from spring-flowering trees. Spatial variability of pollen viability was studied on pollen sampled from thirteen trees selected at four sites in 2011: two trees at the site of Košutnjak, three at Banovo Brdo, five at Ada Ciganlija and three at Bojčin Forest. The sample used to study the temporal variability of the analyzed pollen characteristics was collected from the same tree at Banovo Brdo over a period of nine years (2004-2012). Due to certain technical problems, such as the insufficient quantity of collected pollen required for analysis or fungal infection of the pollen, only pollen collected in 2004, 2005, 2006, 2007, 2011 and 2012 was suitable for the analysis. Interindividual variability of pollen viability was studied using the pollen collected from eight trees at the site of Ada Ciganlija in 2004. All the study sites are located in the area of Belgrade: Košutnjak – a forest park of protection categories II and III by decision no. 501-2419/14-C-20, ass. *Orno-Quercetum cerris virgilianae* Jov. et Vuk. *typicum* Tom. 1990 and *Quercetum frainetto-cerris* Rud. 1949; Banovo Brdo – an urban park, ass. *Quercetum farnetto-cerris* Rud. 1949; Ada Ciganlija – an urban forest park ass. *Quercetum farnetto-cerris* Rud. 1949 and *Fraxino angustifoliae-Quercetum roboris* Jov. and Tom. 1979; and Bojčin Forest – a suburban forest park of protection categories II and III by decision no. 501-146/13-C-20, ass. *Carpino-quercetum roboris* Rauš 1969.

Supporting information for examining climate impact

The City of Belgrade (N 44° 49’ N 20° 27’) lies in the humid continental climate zone, with a uniform distribution of rainfall throughout the year. The study of climate impact was based on the data obtained at Republic Hydrometeorological Service of Serbia (RHMSS) weather stations located in the vicinity of the study sites. The sites of Košutnjak, Banovo Brdo and Ada Ciganlija are located within a radius of a few kilometers (5 km) and belong to the weather station of Košutnjak. The site of Bojčin Forest is 25 km away from these sites and belongs to the weather station of Surčin. We analyzed the data on average annual air temperature and annual rainfall, as well as the temperature and rainfall for the months before pollination (January-April).

Experimental procedure and determination of pollen characteristics

Pollen grains were collected from 40-60-year-old trees on a windless morning in the third/fourth week of April. They were collected from the south-facing crown aspect, directly from male flowers – catkins at the stage of full maturity (i.e. pollination). The collected material was purified in the laboratory through a series of sieves to the final diameter of 0.10 mm and then dried for 48 h [31].

Pollen grain viability was determined by the germination rate (%), and the energy of germination represented by the pollen tube length (μm) was studied by a modified *in vitro* method [32]. MS medium with six sucrose concentrations (0, 5, 10, 15, 20 and 25%) in distilled water was used for the purpose of pollen germination [33]. According to unpublished research results, the optimum medium is 15% sucrose and the obtained results refer only to this concentration. The germination experiment was set up in a chamber with the day/night light conditions (16/8 h) and room temperature of 23°C [34]. In order to ensure sufficient moisture, the bottom of Petri dishes was covered with distilled water and the dishes were covered to prevent evaporation of liquids and pollen-grain drying. Pollen grains were sown in a drop of culture medium, in three repetitions. The germinated pollen grains were counted and the length of pollen tubes was measured 24 h after the experiment was established. Pollen grains were considered as germinated when the pollen tube length exceeded half of the pollen grain diameter [35]. This criterion was also applied to the measurement of the pollen tube length. The germination rate was determined by counting the number of germinated pollen grains out of the total number of pollen grains in the microscope field of view (about 100 grains/field). The length of pollen tubes was measured on a random sample of 25 grains in the microscope field of view (about 100 grains/field) within the same test used in the study of pollen germination rate. The samples with less than 5% of germinated grains were treated as samples with no germination. Measurements were done using a Leica Galen III light microscope (magnification x100) [36,37], with a camera (CCD Camera Topica TP/5001).

Statistical analysis

Statistical analysis of data provided parameters of descriptive statistics, mean values of germination rate (%) and the length of pollen tubes (μm). A normality test showed that the obtained values of the studied characters did not have a normal distribution. In order to achieve a normal distribution of data and perform further statistical analyses to assess variability factors, the main data were transformed using adequate transformations (for length of pollen tubes, Box-Cox transformations, and for percentage of germination, Arcsine transformation). The applied models of the analysis of variance (ANOVA) used years and sites as fixed factors and individuals, i.e. trees, as random factors. The results of the statistical analysis were obtained using statistical software packages SAS version 9.1.3 (SAS Institute, INC. 2011. SAS/STAT Users Guide, Cary, NC: SAS Institute, Inc) and Statistica 10 (Statsoft Inc. STATISTICA version 10 2011).

RESULTS

Pedunculate oak pollen is oval in a dry state. It swells in a humid environment and becomes spherical. Within 24 h after the experiment had been established, the process of germination began, i.e. pollen tubes penetrated through one of the furrows on the pollen grain (Fig. 1). Most variants of the conducted experiment used a 15% sucrose concentration as the optimum nutrient medium both for the germination rate and the length of pollen tubes. Therefore, we present results only for this medium (the results on pollen viability in other culture media have not been published yet).

Spatial variability

The study of spatial variability included thirteen trees at four sites in a single year of pollen collection (2011). According to the results of ANOVA ($F=0.13$; $p=0.9416$), germination rate and the length of pollen tubes did not significantly differ between the sites, and at the site level their values ranged from 54.0 to 60.1% and from 132.7 to 160.9 μm, respectively (Fig. 2.).

The study of the differences between the sites included the impact of climate (temperature and rainfall) for the Košutnjak and Bojčin Forest sites, which have nearby weather stations. With regards to climate, certain differences could be observed between these two sites, especially in total rainfall. The respective values of annual air temperature and precipitation for Košutnjak were 12.6°C and 470.4 mm, and 12.3°C and 378.3 mm for Bojčin Forest. The respective monthly values of air temperature and precipitation for the months before the flowering (January-April) were

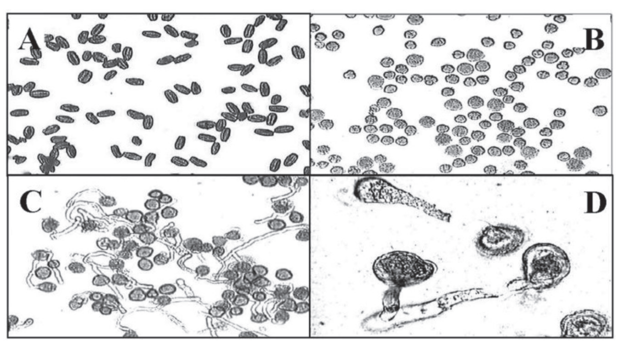

Fig. 1. Pollen grains of pedunculate oak (*Quercus robur* L.). **A** – dry pollen; **B** – swollen pollen, magnification x100; **C** – pollen grain and tube, magnification x100; **D** – pollen grain, magnification 400 x.

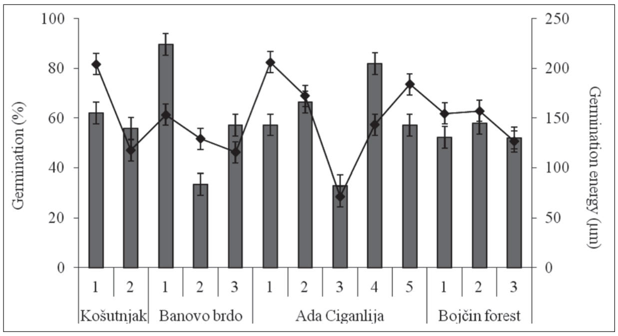

Fig. 2. Relationship between the percentage of germination rate and the length of pollen tubes (µm; full line) of fresh pedunculate oak pollen from different study sites (Košutnjak, Banovo Brdo, Ada Ciganlija, Bojčin Forest) in 2011. The numbers above the sites refer to different oak trees.

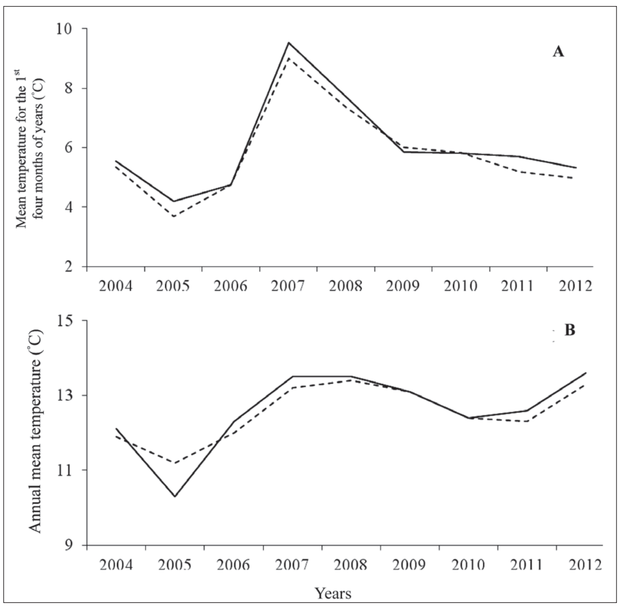

Fig. 3. The mean air temperature for the first four months of years 2004-2012 (**A**) and the annual mean (**B**) between Košutnjak (dotted lines)) and Bojčin Forest (full lines) (2004-2012).

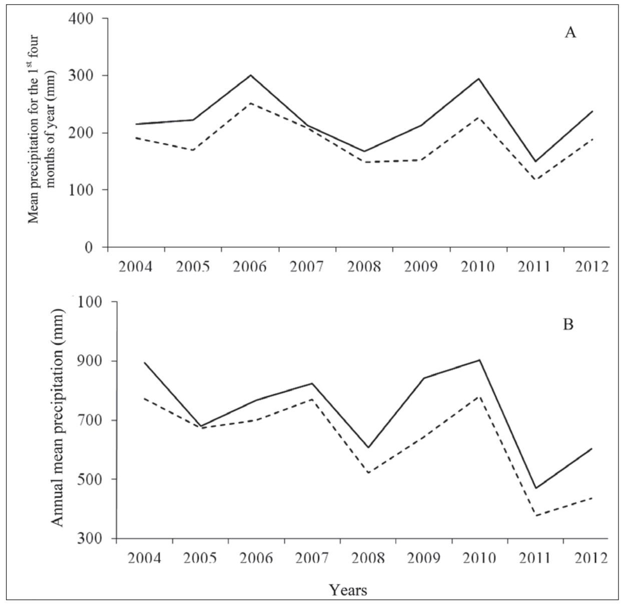

Fig. 4. Climate differences in mean precipitation (mm) for the first four months of year (**A**) and the annual mean (**B**) between Košutnjak (dotted lines) and Bojčin Forest (full lines) sites (2004-2012).

5.7°C and 149.7 mm for Košutnjak and 5.2°C and 116.9 mm for Bojčin Forest. It was confirmed that Košutnjak had greater precipitation in the other years of pollen collection as well (Figs. 3 and 4). The finding that the pollen collected at Košutnjak had higher values of the studied characteristics of viability (germination rate 59.0%, length of pollen tubes 160.9 µm) compared to the pollen grains from Bojčin Forest (germination rate 54.0, length of pollen tubes 146.2 µm) can be directly related to the warmer and more humid climate prevailing at this site. Although the presented results point to the connection between the climatic factors and pollen viability, and to the differences in climate between the sites, the applied model of ANOVA did not confirm statistically significant differences in the analyzed characteristics of pollen viability between the sites.

Statistically significant differences in pollen tube length ($F=10.84$; $p<0.0001$) were found between individual trees of the study sites, thus confirming statistically significant interindividual variability. The largest range of pollen tube length variability was found at Ada Ciganlija (71.3-206.2 µm) and at Košutnjak (117.4-204.3 µm). A wide range of germination rate was recorded at the sites of Banovo Brdo (33.5-89.7%) and Ada Ciganlija (32.8-81.8%) (Fig. 2), but no statistically significant interindividual variability in pollen germination rate was confirmed ($F=1.58$; $p=0.1748$).

Temporal variability

The small time scale of nine years (the scope of research: 2004-2012) confirmed a statistically significant difference between the years for both parameters – germination rate and pollen tube length ($F=13.13$; $p<0.0001$, $F=4.46$; $p<0.0001$), studied on one tree at the site of Banovo Brdo. Pollen grain germination rate and pollen tube length varied widely with the year of

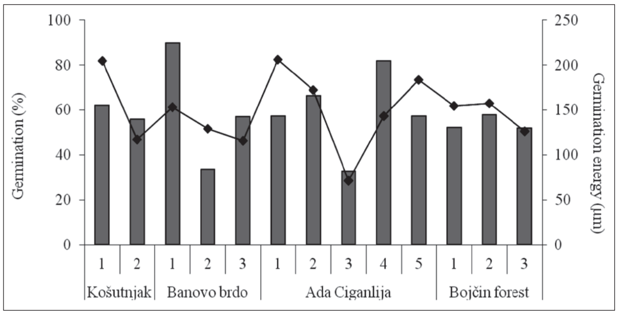

Fig. 5. Relationship between the percentage of germination rate and the length of pollen tubes (μm; full line) of fresh pedunculate oak pollen collected at the Banovo Brdo site (2004-2012). The numbers above the sites refer to different oak trees.

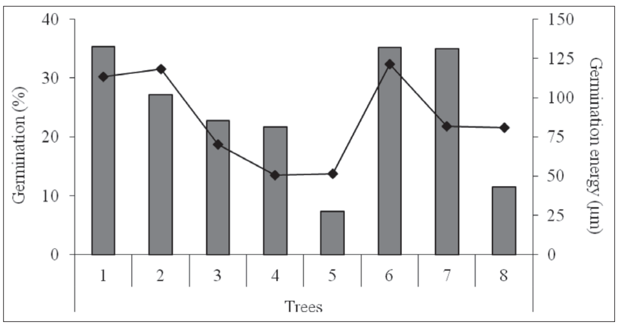

Fig. 6. Relationship between the germination rate and the length of pollen tubes (μm) (full line) of fresh pedunculate oak pollen grains collected from different trees (indicated by the numbers) at the Ada Ciganlija site in 2004.

pollen collection. The lowest value of germination rate was registered in 2005 (28.2%) and by far the highest in 2011 (89.7%). The values of pollen tube length ranged from 81.8 to 196.1 μm, with the lowest value in 2004 and the highest in 2006 (Fig. 5).

As stated above, the lowest pollen germination rate was in 2005. The same year recorded the lowest average air temperature both at the annual (10.3°C) and monthly levels (4.2°C) in the months before pollination.

Individual variability

Interindividual variability of pollen viability was studied on pollen collected in 2004 from eight trees at Ada Ciganlija. Germination and pollen tube length values were in the range of 7.3-35.3% and 50.8-121.5 μm, respectively (Fig 6). Statistically significant differences between the trees for both studied characteristics of pollen grain viability (germination rate and pollen tube length) were confirmed by ANOVA ($F=3.03$, $p<0.0316$; $F=22.49$; $p<0.0001$, respectively). The obtained germination rate values were the lowest for tree no. 5 (7.3%) and the highest for tree 6 (35.3%). The pollen tube length values ranged from 50.8 μm (tree 4) to 121.5 μm (tree 6).

The data on temperature and precipitation for 2004 (pollen collection year) did not differ significantly from the other study years, which might otherwise have an impact on the reduced average pollen germination (Figs. 3-5).

According to the results, the average values of the viability of fresh pedunculate oak pollen (germination and pollen tube length) are as follows: 58.2% and 148.9 μm for the study sites, 54.7% and 144.1 μm for the pollen collection years and 24.5% and 86.1 μm for individuals, i.e. trees.

DISCUSSION

Pollination, fertilization and maturation of pollen are complex processes. In oaks, these processes take about two years and they are affected both by environmental and individual factors. The obtained results confirmed interindividual variability of pollen viability (germination rate and the length of pollen tubes). Statistically significant differences between the years of pollen collection further confirmed the temporal variability, while the spatial variability (differences between sites) was not statistically significant, according to results of the ANOVA test. Contrary to our results, research by Schueler et al. [38] confirmed the significance of local environmental conditions, stressing that the low initial germination rate of pedunculate oak pollen (24.8-65.0%) was related to its sensitivity to sunlight. In our study, pollen germination and energy of germination were not significantly different between the study sites. However, a higher pollen germination rate was recorded at the Košutnjak site compared to that at the Bojčin Forest site. According to the annual values of climatic factors and values for the months before pollination (January-April), Košutnjak had significantly higher amounts of rainfall than Bojčin Forest (470.4 mm and 149.7 mm vs. 378.3 mm and 116.9 mm, respectively), which confirms the impact of precipitation and temperature on the quality of pollen. Similar results were obtained previously [39], when lower amounts of rainfall at the stage of flowering caused a lower rate of pollen germination in *Campanula bononiensis*. Furthermore, a negative correlation between temperature and germination of pollen (the higher the temperature, the lower the percentage of germination) and a positive correlation between precipitation and pollen germination (the heavier the rainfall, the higher the percentage of germination) were confirmed in several *Campanula* species [40]. Other studies have also confirmed the favorable impact of higher temperatures on an increase in pollen germination [41,42]. Temperature has significant effects over the entire period of flower formation, which in pedunculate oak starts in one year and is complete in the following. This study focused special attention on the fact that pollen is particularly sensitive in the stages immediately before and during maturation, when the process of meiosis takes place, the first flower buds open and male catkins elongate [43].

The finding that the germination rate was lower in the year with the lowest temperature and higher at the site with more precipitation and higher temperatures is consistent with the literature, stressing the significant effect of the pollen collection year and climate on pollen viability. The literature points to a wide range of germination variability (5.9-90.7%) of fresh pedunculate oak pollen [30]. The interindividual variability within the same year and between different sites confirmed by our study points to the impact of local microclimate conditions on pollen viability traits. The differences between individual trees and future study of these differences within a specific time scale could help us in the selection of trees that will be included in inbreeding programs as donors of good-quality viable pollen.

This paper marks the beginning of long-term and extensive research aimed at obtaining patterns of pollen variability under the influence of climate factors, which exhibit strong changing trends from year to year. The results obtained on the individual variability of pedunculate oak pollen and the impact of environmental conditions on the quality of pollen have a practical role in the selection of pollen donor trees and in the breeding programs of this important species.

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Authors' contribution: Branislava Batos conducted material sampling, experimental research and presentation of the results. She organized, prepared and wrote the paper. Danijela Miljković statistically processed data and participated in the presentation of the results and paper writing.

Conflict of interest disclosure: The authors state that there is no conflict of interest regarding the publication of this article.

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