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SOME CHARACTERS OF THE POLLEN OF SPRING AND SUMMER FLOWERING COMMON OAK (*QUERCUS ROBUR* L.)

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Abstract - Summer flowering of the common oak is a natural rarity, considering the large area of the species natural distribution. This phenomenon can be classified as an ancestral (atavistic) property. Pollen morphological and physiological characters from spring flowering male inflorescences collected over the period 2004 - 2007 and summer flowering male inflorescences collected in 1999 were compared. The analysis included the pollen of a tree with frequent summer flowering and a control tree with spring flowering only. The size and form of summer pollen differed from the spring flowering pollen**.** The germination percentage and germination energy depended on the pollen growing medium (0, 5, 10, 15, 20, 25% sucrose solution), year of collection (2004 - 2007), temperature (+5°C and -20°C) and storage period (1 to 24 months). The study results are significant for the explanation of common oak phylogenetic development, and they contribute to the knowledge of pollen characters under the effects of different factors.

Key words: *Quercus robur* L., spring and summer flowering, pollen, morphology, germination percentage, germination energy, storage

INTRODUCTION

The Common Oak (*Quercus robur* L.) occupies the largest area of distribution in Europe of all *Quercus* L., (fam. Fagaceae) species. In Serbia, the common oak is an autochthonous, widely distributed species, particularly in Vojvodina, and it is the most represented tree species (Šoškić, 2006) after beech and sessile oak. Its adaptation to different site conditions has resulted in a great number of forms and varieties of the species. Individual differences are often greater than the differences from other species of the same genus (Čanak et al., 1981; Kleinschmit et al., 1995; Bodens et al., 1997; Ponton et al., 2004; Bašić et al., 2007).

Quercus species are characterized by a complex annual shoot (Gruber, 1992). The polyphase formation of annual shoots has been studied by many authors for: *Q. cerris* L. (Janković, 1956; Bobinac and Vilotić, 1998), *Q. robur* L. (Bobinac, 1994; Franjić, 1996), *Q. petraea* (Matt.) Liebl. (Ponton et al., 2004), *Q. virgiliana* Ten. (Bobinac et al., 2000). Common oak growth is manifested in different phases of ontogenetic development (Bobinac, 1994). There are significant differences between the growth phases in leaf and shoot morphology, anatomy and physiology (Masarovicova, 1991; Borzan, 1993; Ponton et al., 2004; Broshtilov, 2006). Polyphase growth can be induced by mechanical injuries, changeable conditions of environmental factors, primarily climate, and their synergistic effect with biotic factors (Sabatier et al., 2003; Girard et al., 2010; Bobinac, 2011). It is supposed that this process is based on the interaction of genotype and environmental factors, i.e. the capacity of common oak ontogenetic adaptation to different environmental conditions (Bobinac, 2011).

The unusual transformation in flowering was the basis for the study of pollen morpho-physiological variation. Pollen morphology is often applied in systematics and paleopalynology (Ćalić-Dragosavac et al., 2009; Makino et al., 2009; Naryshkina and Evstigneeva, 2009). According to Liu et al. (2006), the identification of oak pollen is possible mainly at the genus level; pollen of the genus *Quercus* (21-44 x 17-40 μm) is smaller than that of *Fagus* (32-40 x 35-46 μm) and larger than pollen of the genus *Castanea* (14-23 x 11-17 μm). It was reported that, based on the structure of pollen grain exines, it is possible to separate some types of pollen which are more frequent in evergreen or deciduous oaks, but the absolute identification of fossil pollen is not possible. Solomon (1983), based on palynological analysis of red oak pollen morphology from the eastern North America, points out a high intraspecific variation and low interspecific variation and an uncertain taxonomic status. Gomez-Casero et al. (2004), based on pollen analysis of four Mediterranean oak species in Spain, confirmed that the pollen form characterizes the genus, and pollen size characterizes the species.

A preliminary control of pollen quality is required because not all trees of a species in a population produce good-quality pollen (Kormutak et al., 1994; Grbović and Isajev, 1997). Significant factors of successful pollination are the phenological uniformity of trees (Franjić et al., 2011), intraspecific variation in pollen germination percentage, as well as the effect of external factors on pollen quality (Vuletić, 1973, Batos and Nikolić, 2004).

A sufficient quantity of good-quality pollen is a precondition of controlled pollination, which is one of the most reliable methods for the analysis of the genetic traits of the selected individuals and their combining ability (Boavida et al., 2001). In addition to the above, limiting factors can also be the spatial and temporal isolation, as well as parental incompatibility. The overcoming of the above limitations requires an in-depth study of pollen morphology and physiology, and the conditions of pollen viability preservation (Nikkanen et al., 2000). Most papers dealing with the preservation of pollen viability report on the results of viability after a period of storage, and not on the changes during the storage period. A successful method of pollen viability conservation is cryopreservation (Jensen, 1970; Kirby and Stanley, 1976; Cram and Lindquist, 1984; Morucchio et al., 1990; Lanteri et al., 1993), storage in liquid nitrogen (Connor et al., 1993) or vacuum (Shoenike and Stewart, 1963). Bearing in mind the periodicity and variation in the abundance of oak flowering and pollen yield, the availability of good-quality pollen depends on storage conditions (Gomez-Casero et al., 2004).

The unique transformation in common oak flowering makes it possible to determine the potential differences in pollen size and form in summer and spring flowering, and the collected spring pollen from different years makes it possible to find the optimal growing medium for fresh and stored pollen and to define the conditions of preserving its viability within a two-year period.

MATERIALS AND METHODS

Common oak pollen was collected from a tree observed for summer flowering (tree 1) and a neighboring control tree (tree 2), in which summer flowering had not been detected to date. The trees were adjacent in the City Park at Banovo Brdo (Belgrade), they were approximately of the same age, size, and crown form, and in full physiological maturity. Polyphase formation of annual shoots was frequent in both trees, but with different intensities from year to year. Summer flowering pollen (from the second phase) was collected from tree 1 only in one year (1999), and spring flowering pollen was collected from both trees during four successive years (2004, 2005, 2006 and 2007).

Pollen was collected in the field directly from male flowers – catkins, which were in the stage of pollination. It was purified and desiccated in the laboratory at a temperature of 30°C/48 h. From the same sample, one part was used immediately for the analysis of the morphological and physiological traits of the fresh pollen. The remaining part was stored in flacons, in an exicator with Silica Gel, at the fixed temperature treatments, and applied in the analysis of stored pollen viability.

The analyzed morphological traits included measured values, pollen grain length and width (μm) and calculated value, and pollen grain form coefficient (KO=100*width/length). Pollen dimensions were measured on dry pollen, on a sample of 100 pollen grains.

The analyzed physiological traits included pollen viability (germinability, germination energy) of fresh and stored spring pollen collected from trees 1 and 2 in 2004, 2005, 2006 and 2007. The preservation of pollen viability was analyzed for a period of up to two years of storage at temperature treatments (+5°C and -20°C). The experiments for the analysis of stored pollen viability were established every month during the first year of pollen storage and every three months during the second year of storage.

Six sucrose concentrations in distilled water (0, 5, 10, 15, 20, 25% solutions) were applied in the assessment of the optimal pollen germination medium for all variants of fresh and stored pollen. The pollen germinated in laboratory conditions at a temperature of 25°C (Vazquez, et al., 1996). Pollen germination was tested by the sitting drop culture method. The plates with cultured pollen grains were placed on metal spatulas in covered Petri dishes whose bottoms were covered with water to ensure the necessary humidity. The germinated pollen grains were counted and the length of the pollen tube was measured 24 h after the experiment was established, on a sample of three drops of each culture. Pollen grains with a tube-length larger than the pollen diameter were considered as germinated (Ali et al., 1998). Germinability was expressed by the percentage (%) of the total number of grains in the microscope field of view. Germination energy was represented by pollen tube length (μm). Pollen tube length was measured on a sample of 25 grains in the same experiment as the analysis of pollen germination percentage. No germination was assessed if the number of germinated grains was lower than 5%.

The data were processed by SAS software program. Mean values, standard deviation and coefficient of variation were calculated by PROC MEANS procedure. PROC GLM procedure showed the statistical significance of the effect of variation factors: period of flowering, collection year, tree, growing medium, storage temperature, and the number of storage months.

RESULTS

Common oak spring pollen in a dry state has an elongated oval shape with three symmetrically arranged upright furrows. In a humid environment, the pollen grains swell, becoming spherical in shape and the pollen tube penetrates through one of the furrows, by which the pollen germination process begins (Fig. 1).

Fig. 1. Pollen germination of *Quercus robur* L. (2005), spring flowering tree 1, base 15 % sucrose solution), the original B. Batos.

Pollen morphological characters

The summer pollen from tree 1 was significantly smaller (27.3/20.5 μm) compared to the spring pollen (38.7-40.1/21.4-23.6 μm) and the spring pollen from tree 2 (38.7-40.5/21.4-24.1 μm). There were also some differences in pollen shape. The summer

Table 1. ANOVA results for morphological features of pollen *Quercus robur* L. (Length, width and shape ratio of pollen grains) with the factors and tree age (A) and flowering (B). A: Comparison of sources of variability, in 2004, 2005, 2006 and 2007 with spring flowering. B: Comparison of pollen from flowering summer of 1999 and pollen from flowering spring for the years 2004-2007 combined.

Source of variation	Length (μm)				Width (μm)			Shape coefficient (%)		
	df	MS	F	p	MS	F	p	MS	F	p
A										
year	3	74,99	9,10	0,0001	211,51	23,73	0,0001	0,12	17,87	0,0001
tree (year)	3	18,86	2,36	0,0700	27,87	3,13	0,0253	0,01	1,44	0,2311
Error	699	7,98			8,91			0,01		
B										
flowering	1	5721,63	661,19	0,0001	130,36	13,12	0,0003	1,38	175,58	0,0001
Error	738	8,65			9,94			0,01		

pollen was more rounded (KO=75.8%) compared to the spring pollen, which was more elongated in tree 1 (KO=53.9-59.1%) and in tree 2 (53.9-60.8%) (Fig. 2). The statistically high significance of flowering period (summer/spring) and the year of pollen collection, as the variation factors of pollen morphological characters was confirmed using the analysis of variance (Tab. 1).

Physiological characters of fresh pollen

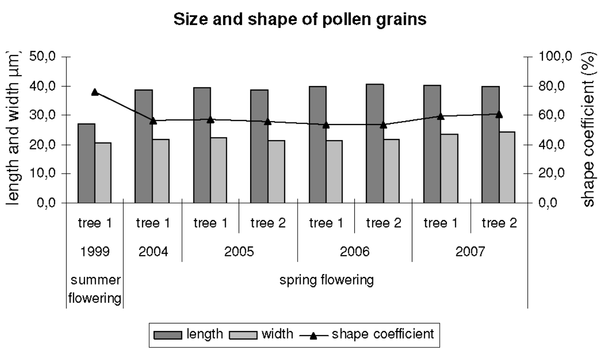


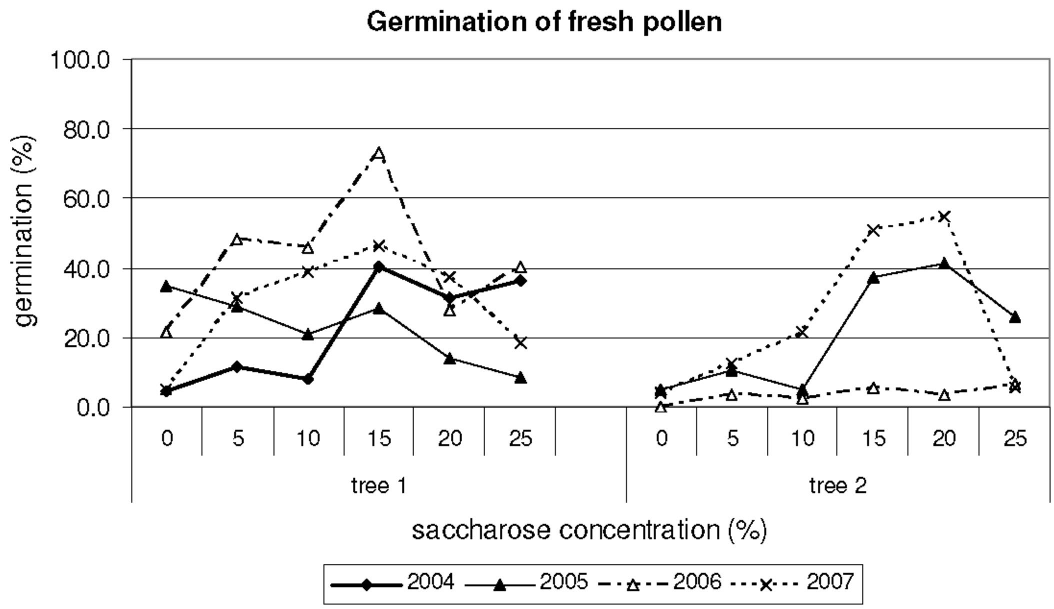
Fig. 2. Morphological characteristics of pollen *Quercus robur* L. (length, width and shape of pollen grains ratio), the pattern of pollen from summer flowering (1999.) and spring flowering in 2004, 2005, 2006 and 2007.

Of the six growth media used for fresh pollen germination, the best germinability (28.2-73.1%) and germination energy (145.1-196.1 μm) in tree 1 was achieved in the 15% sucrose solution. For tree 2, germinability was the best in 20% sucrose solution (41.3-54.8%), and germination energy (70.0-111.4 μm) in 15% sucrose solution (Fig. 3a, b).

Physiological characters of stored pollen

The Common oak pollen stored at +5°C can retain its viability (germinability and germination energy) for a maximum of two to three months. This is followed by rapid decrease in viability. Treatment at -20°C enabled the preservation of pollen viability for up to two years. After 11 months of storage under optimal conditions (temperature treatment -20°C, growing medium 15 and 20%), pollen germinability accounted for 39.1 - 82.9%. After two years it ranged from 24.9 to 46.3%, depending on the year of pollen collection. Pollen from 2006 retained its germinability for the longest time and it also had the highest germinability in the fresh state, which points to the significance of the year of pollen collection. There was an increase during the second and the third months of pollen storage (July - August), and during the winter months (November - January) there was a decrease in germination percentage and germination energy.

Regarding the change in pollen viability during the storage period (months), as well as the storage conditions (temperature treatments), there were no major differences between tree 1 and tree 2. During the storage period, both germinability and germination energy were better at greater concentrations of sucrose in the growing medium (Fig. 4a, b).



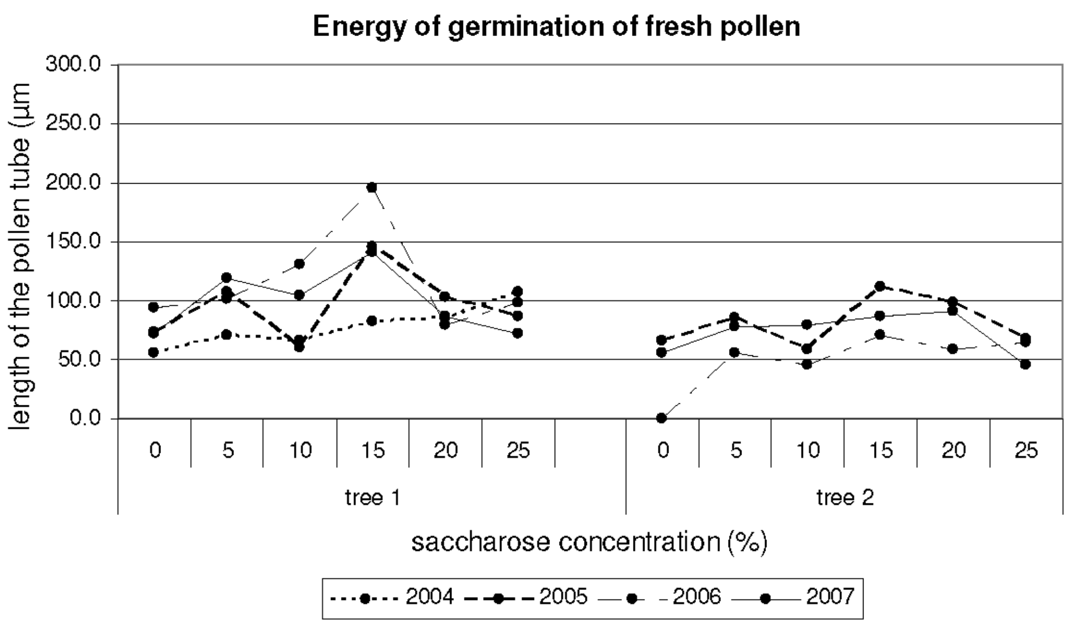
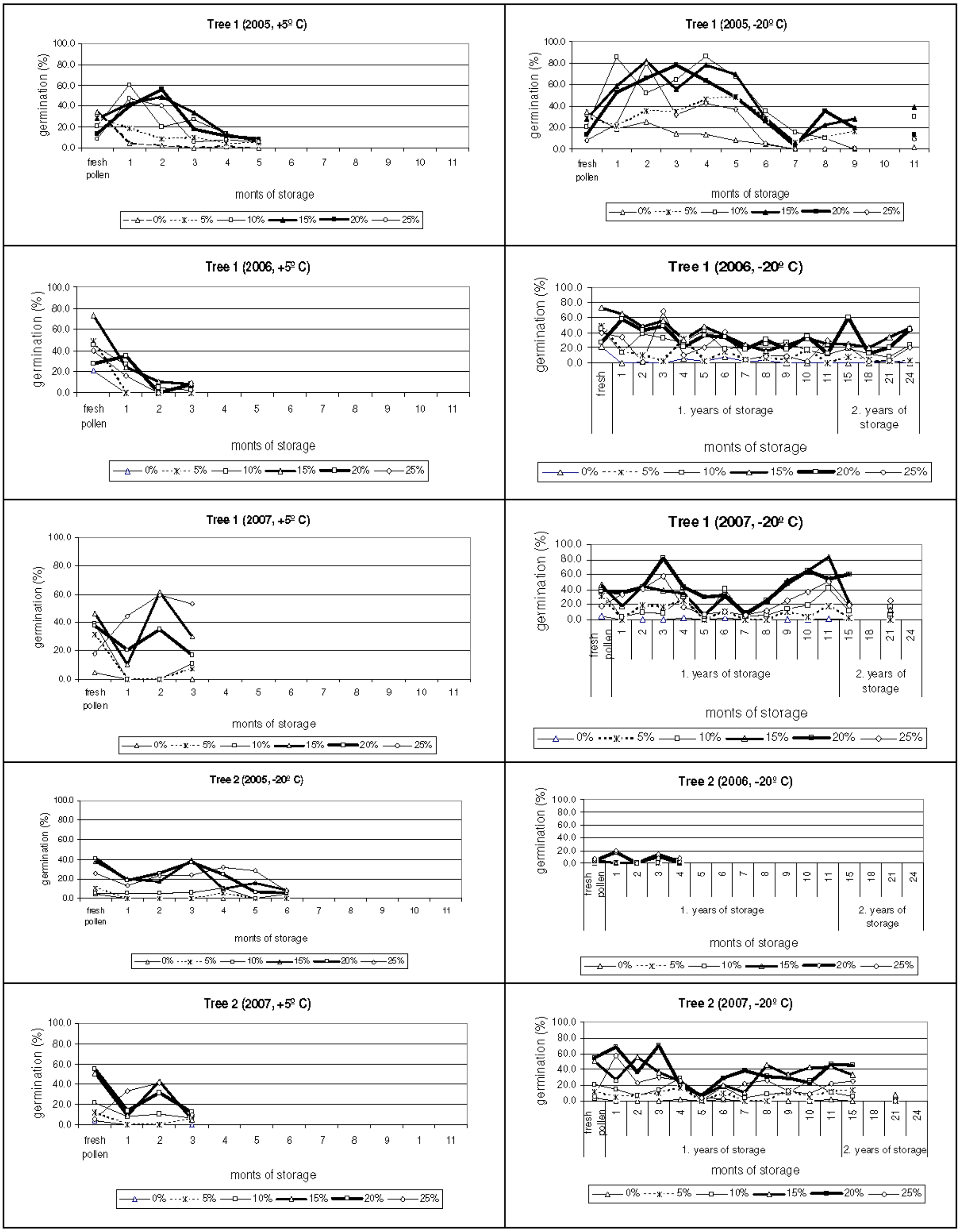


Fig. 3. The percentage of germination and germination energy measured pollen tube length (µm) of fresh pollen of *Quercus robur* L. for 2004, 2005, 2006 and 2007, the six culture media (0, 5, 10, 15, 20 and 25 % sucrose).


Tree 1 (2005, +5º C)
germination (%)
monts of storage
0% 5% 10% 15% 20% 25%
Tree 1 (2005, -20º C)
germination (%)
monts of storage
0% 5% 10% 15% 20% 25%
Tree 1 (2006, +5º C)
germination (%)
monts of storage
0% 5% 10% 15% 20% 25%
Tree 1 (2006, -20º C)
germination (%)
1. years of storage
2. years of storage
monts of storage
0% 5% 10% 15% 20% 25%
Tree 1 (2007, +5º C)
germination (%)
monts of storage
0% 5% 10% 15% 20% 25%
Tree 1 (2007, -20º C)
germination (%)
1. years of storage
2. years of storage
monts of storage
0% 5% 10% 15% 20% 25%
Tree 2 (2005, -20º C)
germination (%)
monts of storage
0% 5% 10% 15% 20% 25%
Tree 2 (2006, -20º C)
germination (%)
1. years of storage
2. years of storage
monts of storage
0% 5% 10% 15% 20% 25%
Tree 2 (2007, +5º C)
germination (%)
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0% 5% 10% 15% 20% 25%
Tree 2 (2007, -20º C)
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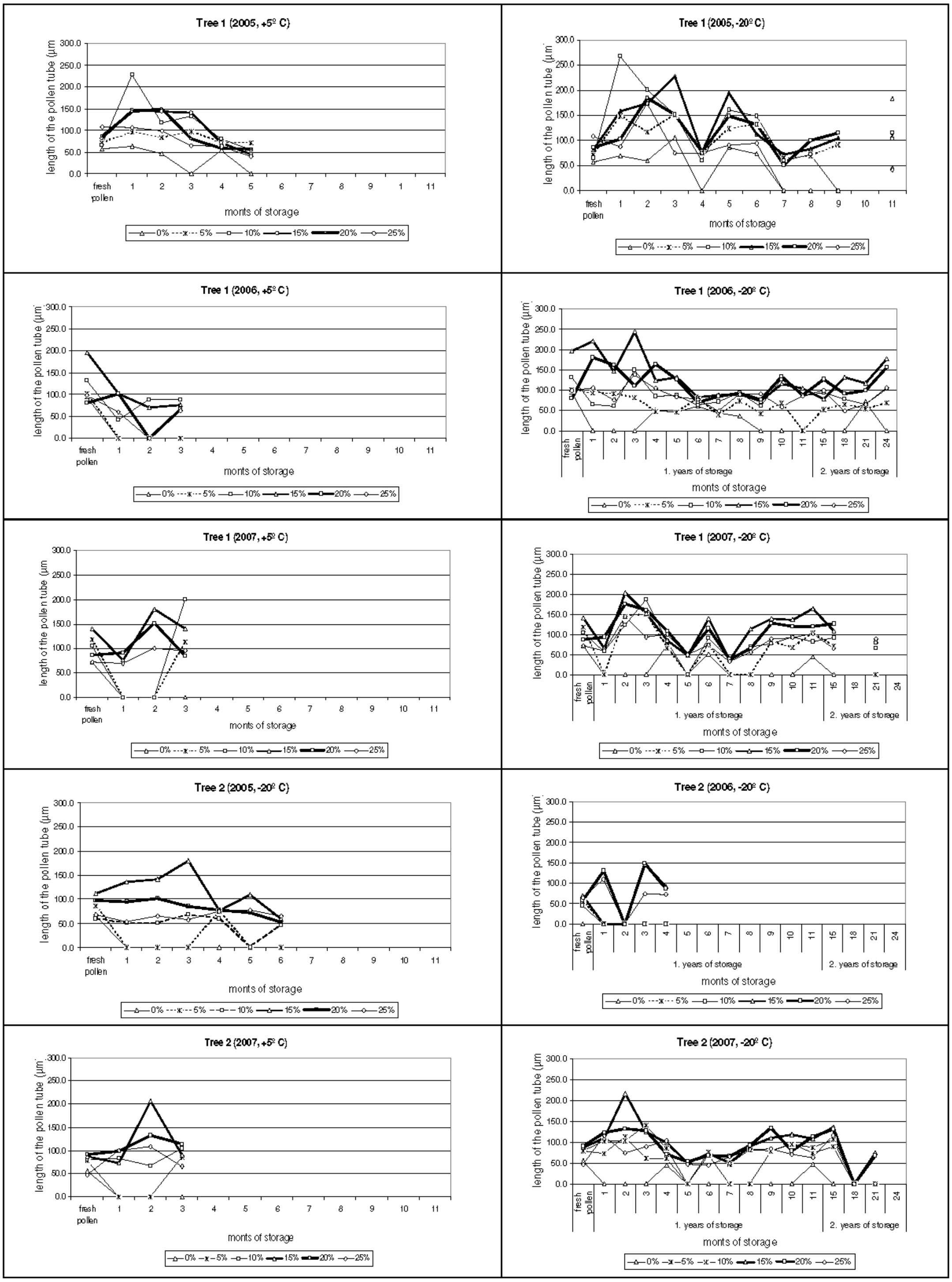

Fig. 4. Germination (a) and pollen germination energy (b) *Quercus robur* L. spring the flowering of the trees (1 and 2) guarded the temperature treatments (5 ° C and -20 ° C), for the months of storage (fresh and 1 - 24 months), nutrient medium (0, 5, 10, 15, 20 , 25 % sucrose) and the collection (2005, 2006 and 2007).

Table 2. ANOVA results for germination energy and germination of pollen *Quercus robur* L. from spring flowering with sources of variation: first collection (2004, 2005, 2006. and 2007.) tree (1; 2), temperature treatments (5 ° C and -20 ° C), months of storage (fresh pollen and pollen stored 1-24 months), nutrient medium (0, 5, 10, 15, 20, 25% sucrose).

Source of variation	Germination energy (%)				Pullen tube length (μm)			
	df	MS	F	p	df	MS	F	p
Year	3	2818,342	25,6	0.0001	3	186348,1	160,63	0.0001
Tree (year)	11	2855,446	25,94	0.0001	11	151230,6	130,36	0.0001
Treatment (year x tree)	10	3187,451	28,95	0.0001	10	119051,9	102,62	0.0001
Months of storage (year x treatment x tree)	65	2146,835	19,5	0.0001	66	133354,3	114,95	0.0001
Nutrient medium (year x treatment x tree x months)	450	812,3678	7,38	0.0001	455	50623,16	43,64	0.0001
Error	1080	110,0972			131,04	1160,12		

The results of the analysis of variance for germinability and germination energy of stored pollen confirm a statistically high significance of all analyzed variation factors; year of pollen collection, tree, storage temperature, months of storage and growing medium (Tab. 2).

DISCUSSION

The flowering of summer shoots is a very rare phenomenon in oaks and it has been described by only a few researchers. In Serbia it was recorded on *Q. robur* (Bobinac, 1994, Bobinac and Tucović, 2005) and *Q. virgiliana* (Bobinac et al., 2000). In Dalmatia (Croatia), the phenomenon of hermaphrodite unseasonal flowering was recorded on one tree of the so-called "green oak" (*Quercus* x *viridis* Trinajstić), which is supposed to be a hybrid of *Q. cerris* and *Q. ilex.* There were no differences in the size and shape between pollen from regular spring flowering and unseasonal summer flowering (Borzan et al., 2000). Contrary to the above-mentioned studies, our research confirmed some significant differences in the size and shape of the pollen collected from spring and summer flowers. Based on previous knowledge, common oak summer flowering cannot be explained only by climate changes. It is assumed that this represents a primitive, ancestral property of the family Fagaceae. The study of summer flowering contributes to a better understanding of intraspecific variation and it has a high significance for the explanation of the *Quercus* species phylogeny (Bobinac et al., 2000; Borzan, 2000). The morphometric analysis of stomata of the common oak tree with frequent summer flowering (Bobinac et al., 2001; Tucović et al., 2002) showed a significantly higher number of stomata, which were also significantly smaller-sized, compared to the control tree with spring flowering only. The identified anatomic differences can be considered as genetically conditioned, as the environmental effect was minimized because the trees were in close proximity.

There are significant intraspecific differences (Shah et al., 2005) in pollen grain sizes (*Quercus dilatata* Royle < *Quercus ballota* Griff. < *Quercus incana* Roxb.) and structure (tricolporate, rounded, reticulate-fine with spaced rods exine sculptures, respectively). The pollen of the *Quercus* species belongs to a group with medium-sized pollen grains. The common oak pollen size is between that of *Q. pubescens* pollen, of which it is larger, and *Q. farnetto, Q. petraea, Q. macedonica* and *Q. cerris* pollen, of which it is smaller. The results of the presented morphological analysis of common oak pollen from the spring flowers of the analyzed trees correspond to the reference results (Erdtman, 1952; Jovančević, 1962; Vuletić, 1973).

The present study confirms significant differences in pollen sizes and shapes depending on the flowering period (summer/spring) and year of pollen collection. Summer flowering pollen is smaller and more rounded than spring flowering pollen. The measured values of freshly collected spring pollen germinability are not high. According to reference data, the low common oak pollen germinability is under the effect of external factors; temperature, photoperiod, water stress, effect of pollutants and their interaction and susceptibility to sun radiation (Gomez-Casero et al., 2004; Shueler et al., 2005). Pollen viability of 40% is considered as a necessary minimum for the successful fertilization of woody species (Callaham and Duffild, after Kirby and Stanley, 1976). The relatively low common oak pollen germinability points to a necessary control of its viability and the need to establish a method for its preservation. The increase in germination percentage in the initial period of storage can be affected by pollen exposure for a short period to a predetermined temperature. The exposure of freshly collected pollen of *Castanea dentata* to a temperature of 4°C for two weeks had a significant effect on the increase in its germination percentage compared to fresh pollen and pollen directly stored at temperatures of -20°C and -80°C. Direct pollen exposure to -20°C significantly reduces its viability, which is in direct correlation with the length of pollen storage (Fernando et al., 2006). Conversely, Caron and Powel (1994) point out an increase in germination percentage after a year of storage at -30°C compared to the fresh pollen germinability of *Pinus banksiana* Lamb. In our study, there was a decrease in pollen germination percentage during storage in the winter months. This phenomenon is referred to in literature as the "winter dormancy of pollen" and it is explained by the decrease in activity and preparation for the following vegetation period. Popnikola (1973), in his analysis of Macedonian pine (*Pinus peuce* Gris.) pollen, found a drop in pollen germination percentage till November and December and then an increase in January, February, March and April during pollen storage at +4°C. The same author reports that Scots pine (*Pinus sylvestris* L.) pollen loses its germinability completely during winter, but the percentage increases gradually with the approach of the flowering period. Jonson (2011) has also pointed to the change in pollen germination percentage in winter months and claims that oak pollen germinability was better after a year of storage at +2°C than in the eighth month of storage.

During the storage period, the optimal medium can be changed, in the sense of better germination on the culture with higher sucrose concentration, which is explained by the changes in structure and the decrease in pollen grain membrane permeability (Lanteri et al., 1993). The significance of the year of collection, i.e. the study differences in common oak pollen viability from different collection years, as well as the reference data (Nikkanen et al., 2000; Kremer and Jemrić, 2006), point to the need of pollen quality control and to the preservation of its viability until the following vegetation.

CONCLUSION

There are significant differences in common oak pollen grain sizes and shapes between spring and summer flowering. There are also differences in pollen morphology depending on the year of collection. Fresh common oak pollen collected from spring flowers has a relatively low viability, but it can be successfully preserved for up to two years under determined conditions. At the temperature of +5°C, germinability can be preserved for only a few months, and at the temperature of -20°C for up to two years. Pollen exposure to -20°C maintained a satisfactory germinability and manifested the stimulation effect during storage. If fresh pollen germinability is high, which corresponds to the year of collection, it indicates the possibility of a longer preservation of its germination percentage. Pollen viability is significantly affected by the year of collection and storage conditions, primarily the temperature and length of storage.

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