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Research Article

Morphological characteristics and antioxidant activity of pollen silver birch (*Betula pendula* Ehrh.)

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ABSTRACT: **Objective:** In Ukraine, as well as in many other countries of the world, different parts of a birch is used in food, pharmacology and cosmetology for the purpose of health improvement. Contrary to this, birch pollen causes an allergy. The aim of this study is to document the morphological differences and antioxidant activity of pollen grains of a silver birch collected from different habitats in Ukraine, and to define the relationship between them. **Materials and Methods:** For the comparative analysis 7 samples of the silver birch pollen have been prepared taking into account different collection sites, background radiation and anthropogenic influence in Kyiv and Rivne regions of Ukraine. The length of polar axis, equatorial axis, apoporial field edge, shape index and other morphological traits were evaluated on scanning electron microscope. Total antioxidant activity of aqueous and ethanol extracts of birch pollen grains were defined in reaction with DPPH. **Results:** Variability of the most important morphological traits of pollen, the length of the polar and equatorial axis, was 17.9 μm (Pereyaslav-Khmelnytsky (control)) – 18.9 μm (Ivankiv) and 22.6 μm (Pereyaslav-Khmelnytsky (control)) – 23.9 μm (Ivankiv) respectively. The shape index ranged from 0.77 (Kuznetsovsk) to 0.81 (Pereyaslav-Khmelnytsky). It was established that silver birch pollen is characterized by high antioxidant activity. A measured value of total antioxidant activity (TAA) for aqueous pollen extracts was between 80.5% (Pereyaslav-Khmelnytsky (control)) and 85.5% (Ivankiv). For the ethanolic extracts it was quantified between 60.3% (Hotsky (control)) and 84.9% (Kuznetsovsk). Statistically significant differences between birch pollen samples from different habitats in Ukraine were found in all researches. **Conclusions:** Our results have confirmed that there are distinctions between populations of *Betula pendula* Roth. in morphological traits and antioxidant activity of pollen grains, and also in the other evaluated traits.

KEYWORDS: silver birch; *Betula pendula* Roth., syn. *B. verrucosa* Ehrh.; pollen; morphology; total antioxidant activity

INTRODUCTION

Silver birch (*Betula pendula* Roth., syn. *B. verrucosa* Ehrh. (name verified on 10-Aug-1998 by ARS Systematic Botanists)) is the most widespread species of the genus *Betula* in the world. It is a beautiful park tree, with ornamental openwork crown, gracefully drooping shoots, fragile leaves and various veins. The birch is extended in all climatic zones, except tundra, reaching 73º northern latitude. It has a wide range in the European part of Russia, growing in Western Siberia, the Altai and Caucasus. Birch also grows throughout most of Europe, except the Iberian Peninsula, North Africa, the Forward and Central Asia. Of all the species of birches, *Betula verrucosa* Ehrh. has the largest area.[1]

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Birch has been used traditionally for medicinal purposes: infusions of birch buds and leaves were used as a diuretic, as an antibacterial agent and for healing wounds.[2,3] Oil extracts of birch buds were also used as a dermatological agent.[2] Birch-tar is a traditional preservative and disinfectant, and its bark is diuretic and febrifuge.[3] Tablets of the absorbed birch carbon are used as adsorbent at an intoxication with poisons and bacterial toxins, at flatulence. Fragrant twigs of silver birch are traditionally used in the medical and preventive purposes in Russian bath. Birch twigs render beneficial effect on the circulatory system, hidrosis, also removes the deleterious substances from the body, perfectly clean skin and the body.[2] It was thought that the birch smell cures melancholy and helps from an evil eye, and the unique birch sap, collected in special days in March and April, purifies the blood.[2,3]

In recent decades, special attention has been paid to study birch pollen from which allergenic properties causes hay fever. Although it is known that it is characterized by therapeutic use.[4] Birch pollen is a ready concentrate of various natural vitamins, trace substances, phytoncids and other extremely necessary substances. It possesses bracing, curative properties. It is extremely useful for cancer patients, as it helps normalize the functions of the body and stimulates them.[5] Birch pollen also renders favorable effect on blood. By its action, it is close to the adaptogenic plants like ginseng, which increases the body resistance to adverse environmental conditions, acts as a tonic and stimulant.[5] It is known that the extract of inflorescences of a birch contains substances with thromboplastick activity. In experiments on animals it shows hemostatic action and is considered as perspective haemostatic.[6] The infusion of staminate inflorescences is used in diseases of the heart, stomach ulcers, gastritis, eczema, furunculosis and anemia, emaciation and infectious diseases.[7]

The main causes of allergies induced by birch pollen are factors of natural and anthropogenic origin. Birch is wind-pollinated tree. Its pollen is light and volatile and is released in major quantities and transported long distances. Due to the wide distribution of this species, the birch pollen is registered across all Europe over the flowering season.[8-10] The effects of the anthropogenic factors under the influence of environment factors (especially warming climate, atmosphere more saturated with carbon dioxid), cause changes of the properties in pollen grains.[8,11-14]

This study is focused on revealing differences in the morphological traits of geographically diverse pollen grains and determining the antioxidant activity of birch pollen from different habitats. Each region has specific vegetation with unique antigenic compositions of plants, their modifications under influence of various anthropogenic factors. For Ukraine (a relatively large country with different climatic and geographical areas), it is necessary to define regional birch pollen allergens. About 7% of the Ukrainian population has an allergy to pollen.[15] Among the allergens of wood plants, *Betulaceae* (and birch in particular), are the most prevalent.[8,14] Quality and standardization of diagnostics and therapy depend on the composition of the pollen, which is influenced by regional environmental factors (abiotic, biotic and anthropogenic origin).[16]

MATERIALS AND METHODS

Plant material

Pollen of *Betula pendula* Roth. has been prepared prior to the beginning of anthesis in Kyiv and Rivne region of Ukraine. Birch catkins were dried at room temperature. After drying pollen from each catkin was shook off into a glass container and stored in a freezer.

Collection sites

In total, 7 samples of birch pollen have been prepared, namely: 1 – Kyiv (pollen was collected from the birches growing in the park zone); 2a – Pereyaslav-Khmelnytsky, Kyiv region (pollen was collected from the birches growing near the road) and sample 2b was collected from the birches growing in the park; 3 – Hotsky, Kyiv region (pollen was collected from the birches growing on separate glades among wood); 4 – Ivankiv, Kyiv region in III Chernobyl zone (according to definition of the territory by the Ministry of Ukraine on questions of extreme situations and on affairs of protection of the population from consequences of Chernobyl accident)[17] (pollen was collected from the birches growing near the highways and apartment houses); 5 – Kuznetsovsk, Rivne region in IV Chernobyl zone (located in 380 km to the West from Kyiv) (pollen was collected from birches growing near the wood, and near the highways); 6 – Borodyanka, Kyiv region (pollen was collected from the birches growing near airdrome).

Morphological analysis

Scanning electronic microscope ZEISS EVO LS 15 was used for research of morphological traits. A thin layer of dried pollen grains were placed on the objective table of the microscope on carbon adhesive discs. Morphological traits were measured and estimated on 140 pollen grains, i.e. 20 for each sample. Software AxioVs40 V 4.8.2.0 (Carl Zeiss, Jena, Germany) was used for measurements. Polar axis (P – the straight line between the distal and proximal poles of a pollen grain), length of the equatorial axis (E – pollen width, the distance between the poles in equatorial part of a pollen grain), angle of the outline of pollen grain in polar view, an internal diameter of apertures and length of an apoporial field edge of pollen grains were measured. The ratio of the polar axis to the equatorial axis also was defined (P/E).

Antioxidant activity

Total antioxidant activity (TAA) of the test samples was estimated by the level of free radicals from the reaction with DPPH ($C_{18}H_{12}N_5O_6$, M = 394.33) dissolved in methanol in an amount of 0.025 g in 100 ml, with a sample of birch pollen according to Y.-T. Kao's modified technique.[18] The appropriate amount of distilled water at room temperature and 70% ethanol were used to make extracts. 5 g of pollen of each sample was dissolved in 100 ml of solvent. Then the mixture was stirred with a mechanical stirrer for 2 hours. Optimum extraction time was obtained by a series of experiments. After extraction 0.1 ml of the extract was mixed with 3.9 ml of the DPPH solution. DPPH reacts with the antioxidant in the sample and gets reduced resulting in a colour change from deep violet to light yellow. The change in absorbance was measured at 515 nm using a spectrophotometer (Genesys 20, Thermo Fisher Scientific Inc., USA) after 10 minutes. TAA in percent was calculated by:

$$\%TAA = 100 \cdot [(A_{control} - A_{sample})/A_{control}],$$

where $A_{control}$ – absorbance of 100% methanol solution taken in the reaction;

A_{sample} – absorbance of testing sample.

All the tests were carried out in 5-single replications.

Statistical analysis

Results were evaluated by standard techniques[19] using MS Excel and STATISTICA 6.1. An estimation of reliability was carried out by F-test at 5% and 1% significance value.

RESULTS

Morphological characteristics of pollen grains

According to morphological description of pollen grains of *Betula verrucosa* Ehrh,[20-22] pollen grains are monad, isopolar. Their size varies from 10–25 µm. Shape of birch pollen is spheroidal, equatorial outline is circular. P/E ratio is suboblate to oblate. Aperture type of birch pollen grains is 3-porate, annulate, operculate, oncus. Ectoaperture is pore more or less circular to slightly elliptic, margins smooth and distinct, with a distinct aspis. Endoaperture is pore of similar diameter to the ectopore. Aspides distinctly protruding. Shape of dry pollen is irregular with irregular outline, irregularly infolded. Dry pollen infoldings are irregular infolded but only interapertural areas sunken. Colour of grains is yellowish.

The results of measurements of morphological traits of birch pollen grains are given in Table 1. As can be seen from the data presented in Table 1, the variability of the length of the polar axis is 17.9 (2a) – 18.9 (4) µm; variability of the length of the equatorial axis is 22.6 (2a) – 23.9 (4) µm. Shape index (P/E) has been determined within 0.77–0.81 (data not shown). Variability of the angle of the outline of pollen grain in polar view is 97.6 (2b) – 105.4 (1) deg; variability of the length of the internal diameter of apertures is 2.9 (6) – 3.4 (3) µm; variability of the length of an apoporial field edge is 25.6 (2a) – 27.4 (4) µm.

Table 1 also shows the results for evaluating the morphological attributes data using statistical method ANOVA. The results of the evaluation for all traits proved statistical differences between samples.

Variance analysis showed there are significant differences between the mean values of all test samples of five measured morphological traits. The criterion of the least significant difference (LSD) has been applied to reveal reliable distinctions between the mean values for each trait (Table 1). Significant differences were defined with a significance cut off value of 0.05.

Our length of the polar and equatorial axis data correlate with the literary data (Table 2).

Statistically significant differences were found between samples 2a and 4 (Table 1 and Figure 1). Polar and equatorial axes are the most common and important morphological characteristics of pollen grains, therefore previous studies paid the most attention to it. As for the length of polar and equatorial axis, domination of sample 2a and 4 was established. These results are well illustrated on Box and Whisker Plots (Figure 1). Although it is necessary to consider that differences are shown not in all traits simultaneously.

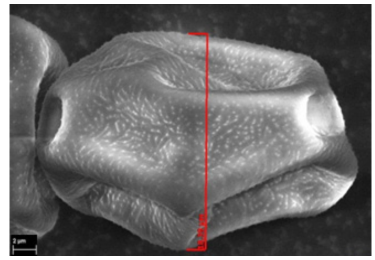
Figure 2 shows variability of studied morphological traits of *Betula verrucosa* Ehrh. pollen grains. Photos made by SEM.

Antioxidant activity of pollen extracts

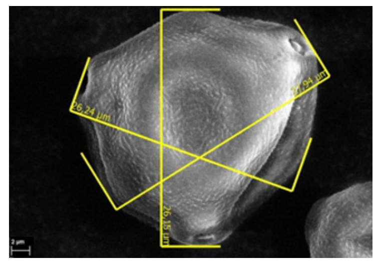
According to the data in Table 3 pollen of silver birch achieved high values of antioxidant activity: values of the TAA for aqueous pollen extracts were defined within 80.5–85.5%, ethanolic extracts achieved 60.3–84.9%. These results show higher antioxidant activity of aqueous extracts of birch pollen in comparing to ethanolic. Table 3 also shows the results for evaluating the antioxidant activity data using statistical method ANOVA. Antioxidant activity is higher in aqueous extracts than in ethanolic extracts.

In previous reports, a comparison of the antioxidant properties of aqueous and ethanolic extracts of different pollen species (mimosa, yucca, chenopod and others)

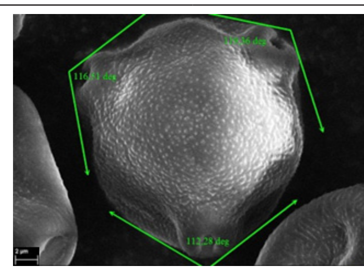
Table 1: Variability and variance analysis of morphological traits of pollen grains of *Betula verrucosa* Ehrh., n=60



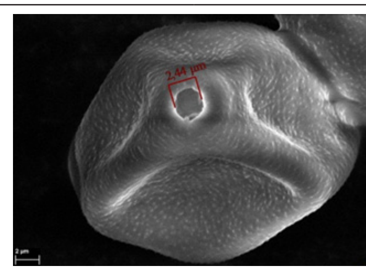
Sample	Length of the polar axis (P), µm						
	min	max	mean	σ	V%		
BV1	16.1	21.2	18.6ab	1.19	6.39		
BV2a	15.2	21.8	17.9b	1.45	8.09		
BV2b	14.3	21.8	18.6ab	1.48	7.99		
BV3	15.1	21.6	18.4ab	1.64	8.92		
BV4	16.5	22.5	18.9a	1.38	7.26		
BV5	15.3	21.1	17.9b	1.36	7.56		
BV6	15.3	21.5	18.3ab	1.44	7.88		
	df	ssq	msq	F	p	Least Significant Difference (Tukey)	
Between grps	6	50.84	8.47	4.17	0.0004	0.05	0.74
Within grps	413	839.34	2.03	-		0.01	0.87



Sample	Length of the equatorial axis (E), µm						
	min	max	mean	σ	V%		
BV1	18.9	26.9	23.3ab	1.92	8.25		
BV2a	16.9	26.4	22.6b	2.20	9.72		
BV2b	19.9	28.2	23.2ab	1.73	7.46		
BV3	19.8	28.1	23.2ab	1.56	6.74		
BV4	20.9	28.1	23.9a	1.66	6.94		
BV5	19.8	26.7	23.3ab	1.34	5.74		
BV6	19.9	27.1	23.4ab	1.44	6.14		
	df	ssq	msq	F	p	Least Significant Difference (Tukey)	
Between grps	6	60.64	10.11	3.44	0.0025	0.05	0.89
Within grps	413	1,214.08	2.94			0.01	1.05



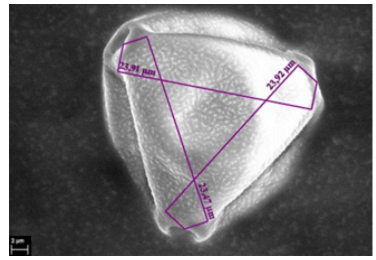
Sample	Angle of the outline of pollen grain in polar view, deg						
	min	max	mean	σ	V%		
BV1	83.8	125.9	105.4a	7.61	7.22		
BV2a	81.3	122.2	99.4ab	8.92	8.98		
BV2b	81.9	110.6	97.6c	6.95	7.12		
BV3	81.2	115.8	98.7ab	7.81	7.91		
BV4	86.6	115.2	101.1ab	6.86	6.78		
BV5	80.8	112.6	98.8ab	6.61	6.70		
BV6	85.6	123.6	103.1a	7.66	7.43		
	df	ssq	msq	F	p	Least Significant Difference (Tukey)	
Between grps	6	2,817.0000	469.50	8.30	0.0000	0.05	3.91
Within grps	413	23,376.3096	56.60			0.01	4.62



Sample	Length of the internal diameter of apertures, µm						
	min	max	mean	σ	V%		
BV1	2.1	4.3	3.3a	0.45	13.62		
BV2a	1.4	4.1	3.0b	0.53	17.56		
BV2b	2.1	3.9	3.1ab	0.45	14.26		
BV3	2.4	4.3	3.4a	0.40	11.62		
BV4	2.5	3.8	3.3a	0.33	10.19		
BV5	2.4	4.1	3.4a	0.34	10.03		
BV6	2.2	4.2	2.9bc	0.43	14.57		
	df	ssq	msq	F	p	Least Significant Difference (Tukey)	
Between grps	6	9.85	1.64	9.1546	0.0000	0.05	0.22
Within grps	413	74.06	0.18			0.01	0.26

(continued)

Table 1:(continued)



Sample	Length of an apoporial field edge, µm						
	min	max	mean	σ	V%		
BV1	22.6	31.1	26.4a	1.92	7.28		
BV2a	20.5	28.7	25.6bc	1.93	7.54		
BV2b	23.5	30.2	26.2ab	1.52	5.83		
BV3	22.5	31.6	26.5a	2.11	7.97		
BV4	22.6	32.7	27.4a	1.83	6.68		
BV5	22.6	29.0	25.6bc	1.54	6.03		
BV6	23.8	30.8	26.8a	1.53	5.71		
	df	ssq	msq	F	p	Least Significant Difference (Tukey)	
Between grps	6	154.31	25.72	8.09	0.0000	0.05	0.92
Within grps	413	1,311.90	3.18			0.01	1.09

n – number of measurements; min – the minimum value; max – the maximum value; mean – arithmetic mean; σ – standard deviation; V – coefficient of variation in%; abMeans within a column with the same letters are not significantly different according to Tukey's multiple range test ($P \leq 0.05$); df – degrees of freedom; ssq – sums of squares; msq – residual mean sum of squares; F – F-test; p – significance level.

Table 2: Comparison of the received data of the main morphological traits of pollen grains of *Betula verrucosa* Ehrh. with literary data

Source	Length of the axis (µm)	
	polar (P)	equatorial (E)
Our data	17.9 – (18.4) – 18.9	22.6 – (23.3) – 23.9
Erdtman G. (1943)[20]	No data	18.6 – (21.8) – 24.3
Pokrovskaya I. (1950)[23]	No data	15.0 – (19.6) – 21.0
Blackmore S. (2003)[24]	17.0 – (19.5) – 24.0	22.0 – (24.5) – 28.0
Polleninfo[25]	21.0 – (22.6) – 25.0	22.0 – (24.0) – 25.0

Data in table in format: min – the minimum value; mean – arithmetic mean; max – the maximum value.

showed that antioxidant activities are generally higher for ethanolic solutions than for aqueous extracts (Table 4), in contrary to our results from wind pollinated species.

Comparison of two solvents used for extraction in this study (aqueous and ethanolic) using variance analysis, has allowed to reveal significant differences between the extractants for all samples, except for sample 5. These results are well illustrated on Box and Whisker Plots (Figure 3). It means that the choice of extractant influences the value of antioxidant activity and only for one pollen sample (5) differences between the extractants was not found. This sample was gathered in another area (Kuznetsovsk, Rivne region in IV Chernobyl zone). This fact testifies the heterogeneity of birch pollen from this place of growth.

DISCUSSION

This work is focused on the identification distinctions of morphological traits and antioxidant activity of pollen grains of *Betula verrucosa* Ehrh., collected from different habitats in Ukraine and in the definition of the relationship between them. The morphological characteristics of pollen grains are the most applicable and indicative for each species, and identification of any morphometric changes suggests the influence of external factors and possible changes in biological properties. Antioxidant activity of pollen extracts is one of numerous indicators of biological activity of living organisms. Moreover, currently evaluation of antioxidant properties of pure natural ingredients, herbal extracts and biologically active admixtures is increasing.[27] Scientists from different countries have reported the antioxidant properties of plant pollens from different species.[18,26-29] It was reported that pollen itself has a high antioxidant capacity.[27] Pollen extracts contain many phenolic components and may block free radicals which are responsible for the development of cardiovascular diseases and cancer.[28-31] A vast number of studies are devoted bee pollen because it is more accessible and diverse in composition. It is confirmed that bee pollen is an important protective factor in the prevention of diseases caused by free radicals.[27]

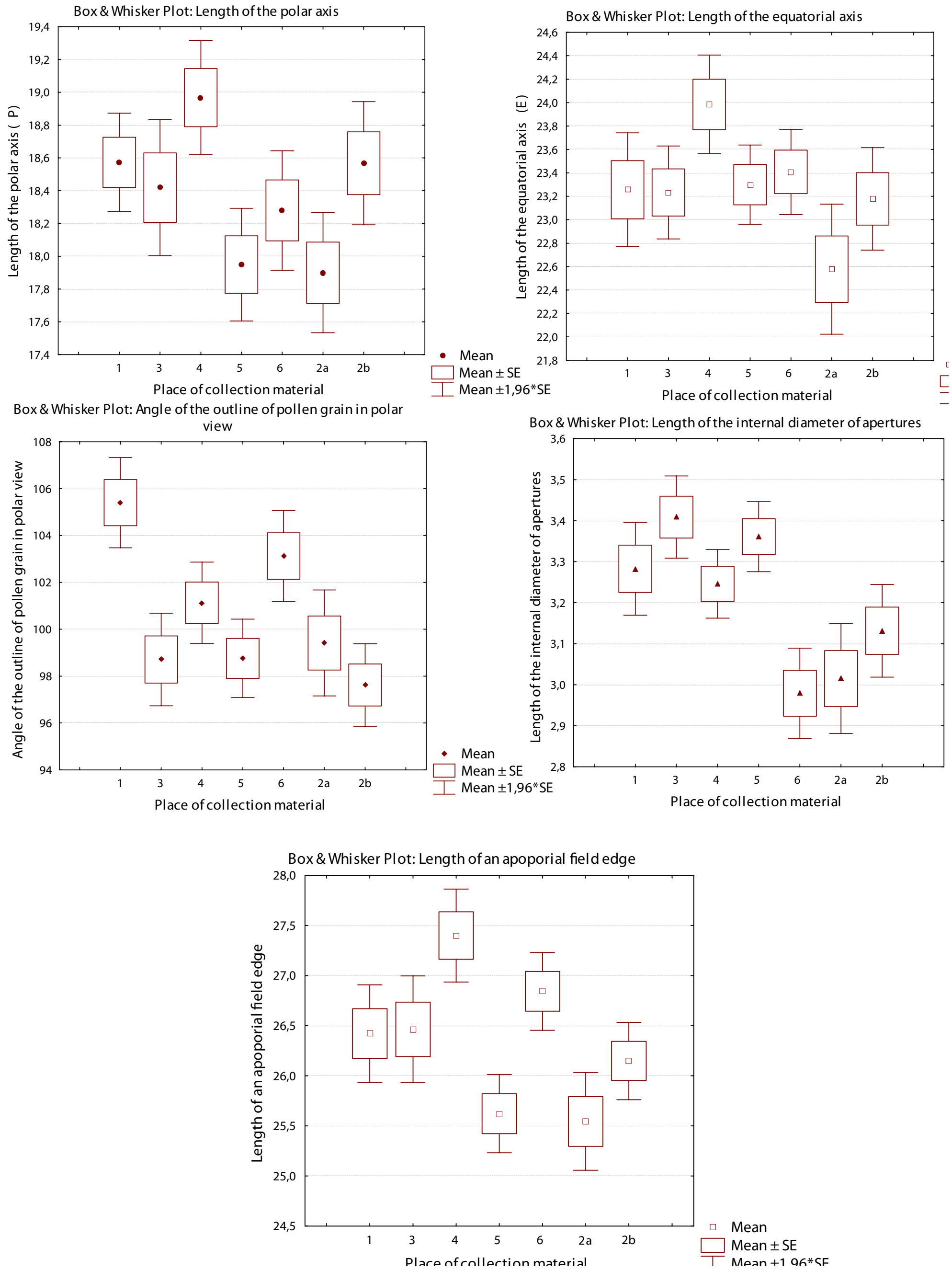


Figure 1. Box & Whisker Plot for five morphological traits of birch pollen.

According to our results, it is necessary to exclude the influence of the following factors:

1. Geographical location of the pollen collection places. All samples were prepared from one climatic zone of the country. Place of growth of the sample 5 is located further north than the rest (51° 27' N), and sample 3 is located further south than the rest (49° 54' N). However, that does not make significant difference.

2. Degree of a maturity and date of collection of pollen. At first pollen was prepared from more southern habitats, therefore we consider that maturity degree has the minimum influence on the results. Pollen of the same plants ripens at different times. This process depends on various factors, such as the meteorological conditions and geographical differences.[32] Therefore, we consider that the growth conditions of birches has the most important effect on the evaluated traits on pollen grains. The safest places of preparation of pollen were samples 2b and 3

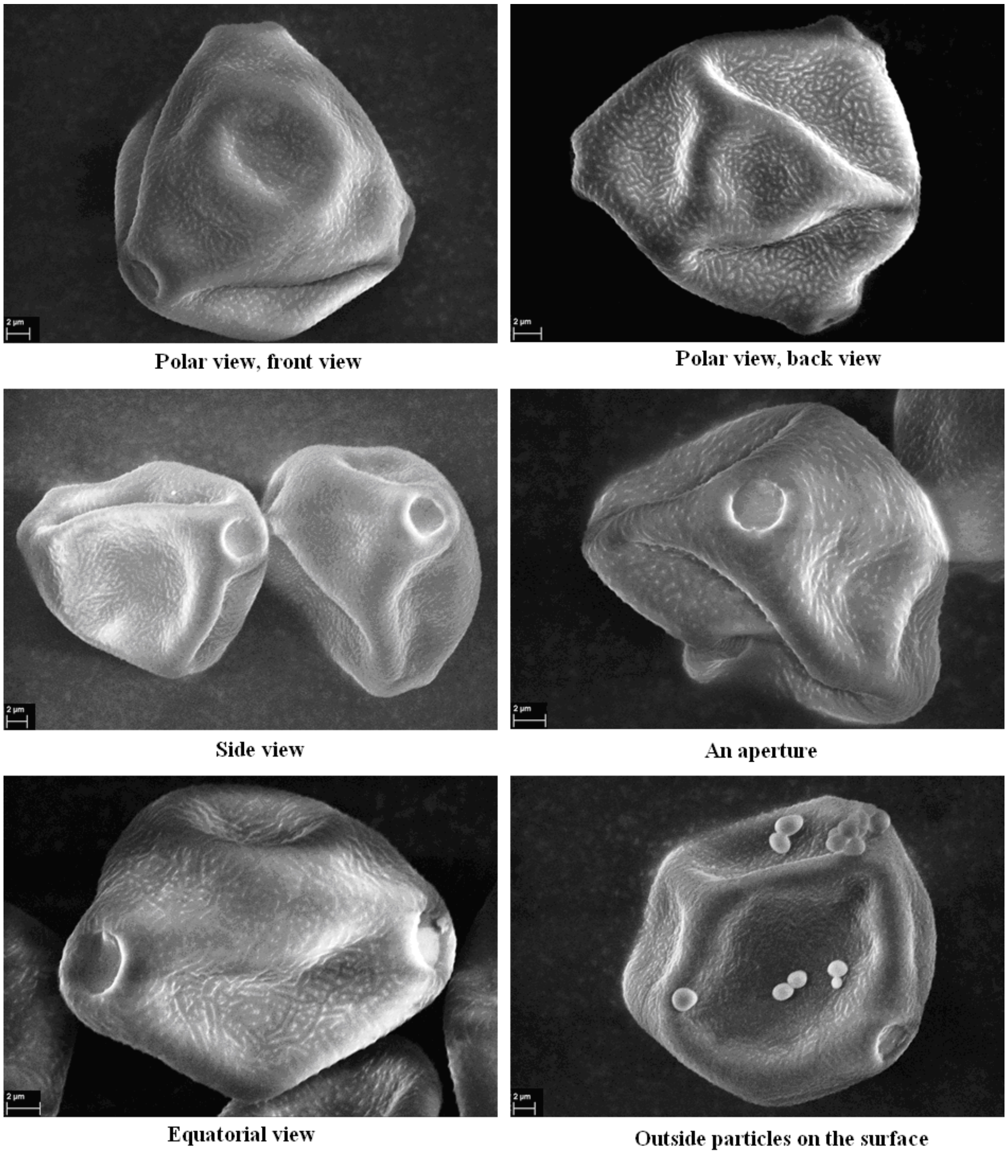


Figure 2. Dry pollen grains of *Betula verrucosa* Ehrh. (Photo: R. Ostrovsky, T. Shevtsova 2011).

(birches grown in the forest and in the nature museum). Particularly, it has not been revealed any significant differences in morphology for the sample 2b by comparison with the other samples. It is possible to state samples 2b and 3 occupy intermediate value among all the other samples of pollen (Figure 1). Samples 2a and 2b were collected from different sites in the same town, but pollen of the sample 2a is collected from the birches growing near the highways and apartment houses that may have influence on birch pollen. Pollen of sample 4 was collected under the same conditions. Samples 2a and 4 are the most different from the other samples (pollen grains from place of collection 2a are the smallest in size and have the smallest value of TAA; pollen grains from place of collection 4 are the largest in size with the highest value of TAA). Such regularity is not observed for ethanolic solutions of these samples. Higher values of TAA in aqueous extracts do not confirm higher values in ethanolic extracts of pollen samples correspondingly.

According to previous studies, pollen is very sensitive to influence of atmospheric pollutants such as industrial and transport emissions.[13,32-34] They registered deposits of pollutants on the surface of pollen, change of the shape of grain. Also it was found that air pollution caused shrinkage, thinning and fragility of pollen. In our case, the smallest size of birch pollen grains is the feature of pollen from Pereyaslav-Khmelnytsky (2a), confirming assumptions of the above-stated authors on influence of atmospheric pollutants on the morphology of pollen.

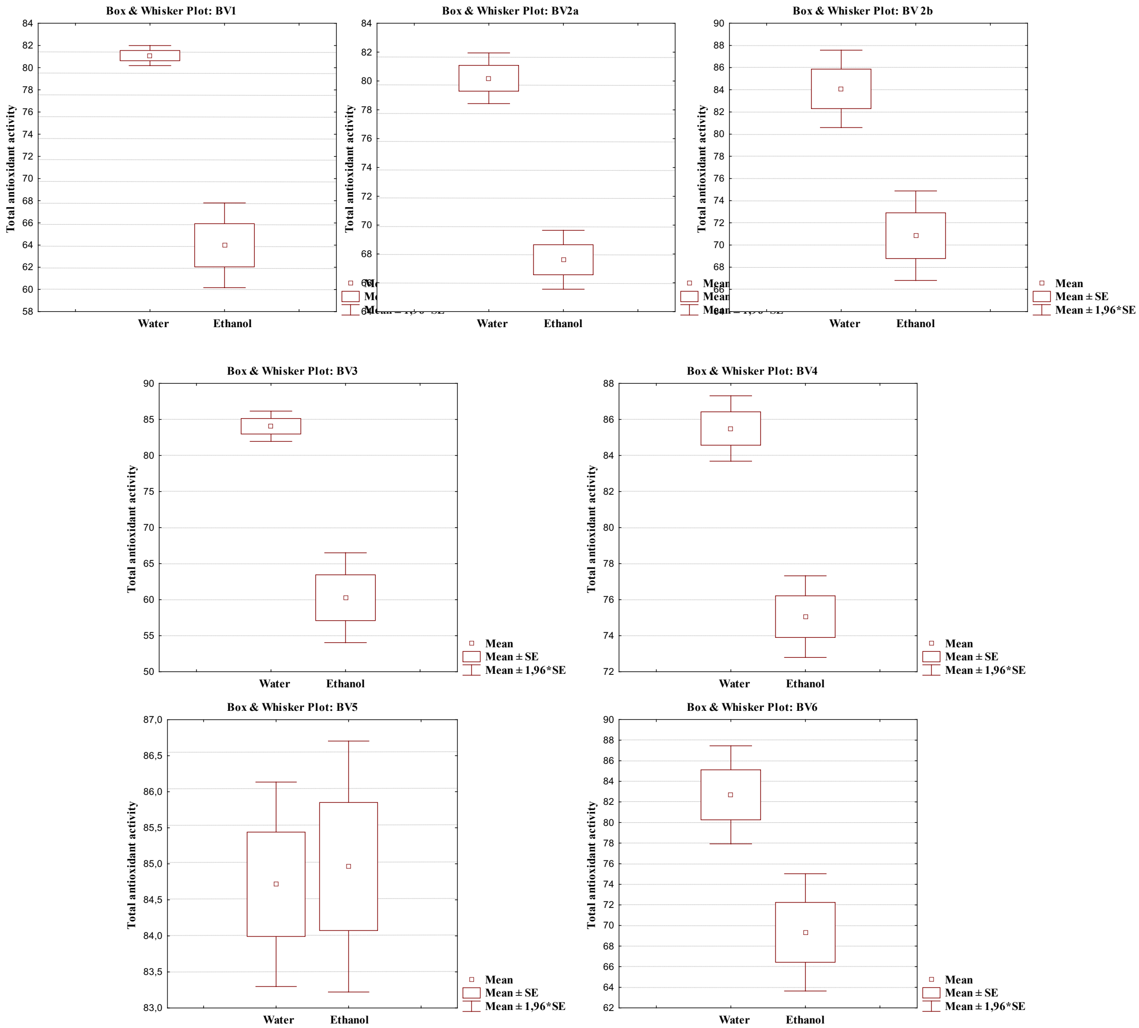


Figure 3. Box & Whisker Plot for the extractants for samples of birch pollen.

As for the high values of the total antioxidant activity, it is difficult to say which substances of birch pollen cause the appearance of properties. In aqueous solution of pollen, hydrophilic substances are responsible for that. Probably it is vitamin C or p-coumaric acid, which was found in not hydrolyzed insoluble residue of *Betula pendula* Roth.[35] It is known that p-coumaric acid is the active antioxidant that blocks free radicals.[36] Besides, one of the functions of p-coumaric acid is stimulation of protein biosynthesis. Pollen allergens by their nature are proteins.[37,38] Probably, high antioxidant activity of aqueous extracts of birch pollen is directly connected with its allergic properties. However, this depends on sensitivity of humans to birch pollen allergens.

CONCLUSIONS

In this paper, the morphological characteristics and the total antioxidant activity of seven samples of pollen of *Betula verrucosa* Ehrh. from different habitats were examined. The study is focused on the comparative analysis among samples in each research as well as determination of the relationship between the morphology of the pollen grains and antioxidant activity. Our results confirmed that there are distinctions in morphological traits and antioxidant activity of pollen grains among populations of *Betula verrucosa* Ehrh. Also, significant differences of the same samples in different studies were found. It was discovered that the smaller the size of the pollen grains, the lower the antioxidant activity of extracts. It was determined that the anthropogenic factor of the place of growth is a major influence on morphology of the pollen and changes in its antioxidant properties.

ACKNOWLEDGMENTS

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Table 3: Total antioxidant activity of pollen aqueous and ethanolic extracts of *Betula verrucosa* Ehrh.,%; n=5 and variance analysis

Sample	Aqueous extract					Ethanolic extract				
	min	max	mean	σ	V%	min	max	mean	σ	V%
BV1	81.2	82.9	81.9	0.67	0.82	61.5	66.5	64.6	1.95	3.02
BV2a	79.4	81.4	80.5	0.90	1.11	66.8	69.6	67.9	1.04	1.53
BV2b	82.4	86.7	84.1	1.78	2.12	68.7	73.6	70.8	2.06	2.91
BV3	82.2	84.9	84.1	1.07	1.28	55.9	63.9	60.3	3.18	5.27
BV4	84.8	87.01	85.5	0.93	1.08	73.5	76.5	75.1	1.16	1.54
BV5	84.0	85.9	84.7	0.72	0.85	83.7	85.9	84.9	0.89	1.05
BV6	78.9	85.4	82.7	2.43	2.94	66.0	73.4	69.3	2.60	3.76

	df	ssq	msq	F	p	F critical
Between grps	1	586,05	586,05	17,87	0,0012	4,75
Within grps	12	393,59	32,80			
Total	13	979,64				

n – number of measurements; min – the minimum value; max – the maximum value; mean – arithmetic mean; σ – standard deviation; V – coefficient of variation in%; df – degrees of freedom; ssq – sums of squares; msq – residual mean sum of squares; F – F-test; p – significance level; F critical – critical values of the F distribution.

Table 4: Comparison of antioxidant activity of *Betula verrucosa* Ehrh. pollen and pollen of other plant species

Plant species	Antioxidant activity (%)		
	Aqueous extract	Ethanolic extract	Source
Betula verrucosa Ehrh.	83.4 ± 1.21	70.4 ± 1.84	Our results
Mesquite	28.6 ± 0.36	57.7 ± 1.31	LeBlanc et al., 2009
Yucca	0.00	18.9 ± 0.69	LeBlanc et al., 2009
Palm	5.7 ± 2.55	11.4 ± 3.46	LeBlanc et al., 2009
Terpentine Bush	10.6 ± 0.00	33.5 ± 1.26	LeBlanc et al., 2009
Mimosa	52.1 ± 3.03	75.9 ± 1.19	LeBlanc et al., 2009
Chenopod	34.9 ± 1.80	54.9 ± 1.36	LeBlanc et al., 2009

Data in table in format: Mean ± Standard Deviation.

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