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The use of fluorescence microscopy for classification of pollen grains

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1. Introduction

A large number of people suffer from a pollen allergy in all regions of the world [1]. In general, pollen allergens are considered a major risk factor for both seasonal allergic rhinitis and asthma [2]. Pollen grains show strong autofluorescence [3]. Fluorescence is non-destructive, sensitive, simple and fast method for analysis of fluorescent compounds contained in very low amounts in the samples [4]. The fluorescence microscopy and fluorescence spectroscopy, in combination with appropriate statistical methods, may provide useful fingerprints in pollen analysis [5].

2. Details of experiment

2.1. Pollen samples

Pollen samples were harvested from Serbian agricultural estate Radmilovac in the early spring 2018. The samples were packed in bags and stored in a freezer at -80 °C until analysis. We analyzed pollen samples of four different botanical species (*Amorpha fruticosa, Robinia pseudoacacia, Rhamnaceae and Rubus* L.).

2.2. Microscopic fluorescence image analysis

Fluorescent microscopic images were obtained by using Axio Observer Z1 Mikroskop, with AxioCamMR3 camera (8 bit per channel). Pollen samples were deposited on glass plates for the measurements. The same optics was used for all recordered pictures, in order to avoid chromatic aberations. The excitation/emission wavelengths were: 358/461 nm (49-DAPI), 488/510 nm (38 GFP), and 558/580 nm (DsRED), in the following text referred to as blue, green (38 GFP) and yellow, respectively. Series of images were captured for each sample, in order to create its representative image with a 10× objective lens. Each image was considered a matrix, where each element represents one pixel. The captured images (Figure 1) were analyzed using ImageJ program to provide the average red, green, and blue pixel values for the fluorescence intensity of each pollen grain selected in an image. Image analysis methods are quantitative tools for analyzing fluorescence and bright-field microscopy data.

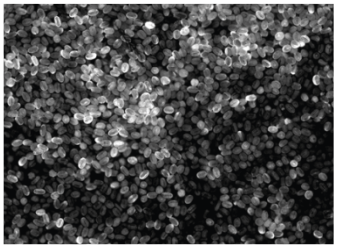
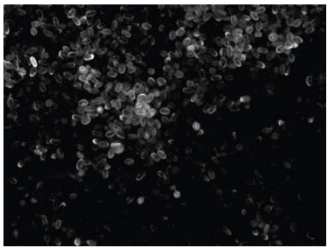
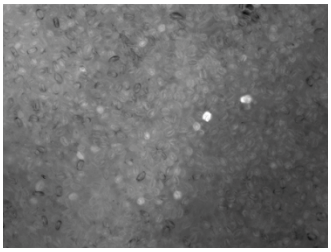
a)  b)  c) 

Figure 1. Microscopic fluorescence image for *Rhamnaceae* pollen grains through a) blue (49-DAPI), b) green (38 GFP), c) red color (DsRED) filters

2.3. Measurement of the fluorescence spectrum

Fluorescence spectra were collected using a Fl3-221 P Fluorolog spectrofluorimeter (Jobin-Yvon Horiba, Paris, France) equipped with a 450 W xenon lamp and a photomultiplier tube. All pollen samples were measured in a front-face configuration of the measuring cell. The illumination's incident angle was set to 22.5 °C, to minimize light reflections, scattered radiation and depolarization phenomena. The Reyleigh masking was applied in order to reduce Rayleigh scattering from the solid sample which limits the sensitivity and accuracy of the measurement. The fluorescence emission spectra in range from 350 to 600 nm, were recorded with excitation wavelengths of 330 to 430 nm. The integration time was 0.1 s, and the wavelength increment in excitation measurements was 5 nm, and emission increment was 1 nm. A spectral band width of 2 nm was employed for both the excitation and emission slits. The average of the 21 emission spectra recorded for various excitation wavelengths, for various pollen samples, are shown in the Figure 2.

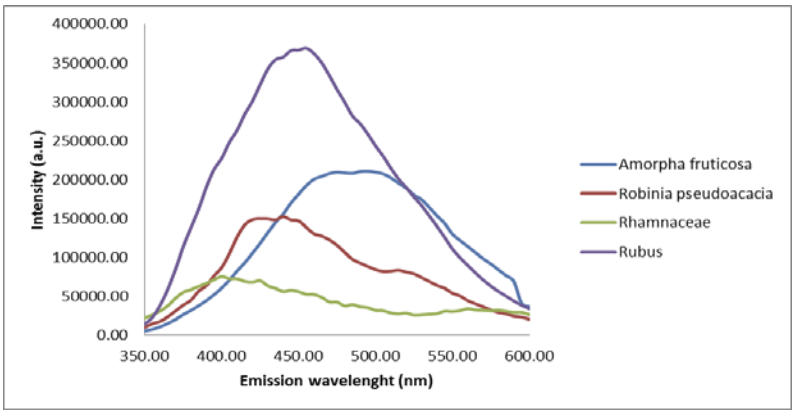


Figure 2. The average fluorescence emission spectra for different pollen samples

2.4. Statistical analysis

Principal Component Analysis (PCA) is a statistical method used to reduce the dimensionality of a data set whilst retaining the information content [6]. Principal Component Analysis (PCA) was performed using the CAMO Software AS package for The Unscrambler X 10.4. All data were group-scaled prior to PCA. The singular value decomposition algorithm (SVD) and a 0.95 confidence level for Q and Hotelling T2 limits for outliers were chosen.

For each sample the brightness results of microscopic fluorescence image or average of the 21 emission spectra recorded for various excitation wavelengths were used as input values in PCA, in order to take into account contribution of all fluorophores present in the sample. The results of PCA analysis are shown in the Figure 3.

3. Discussion

The PCA was used to classify pollen samples according to the differences in microscopic fluorescence and characteristic emission spectra. The scores and loadings plots obtained for pollen samples are shown in Figure 3. The PCA resulted in a two-component model which explains 98 % (microscopic fluorescence) and 99% (fluorescence emission spectra) of total variance. Based on brigtness data, *Amorpha fruticosa* was separated from the other investigated pollen samples along positive PC1, while *Robinia pseudoacacia* was separated along negative PC1. The discrimination of *Rubus* L. sample was along positive PC2, and of *Rhamnaceae* sample along negative PC2. PCA results for fluorescence emission spectra confirm separation of the pollen samples based on their characteristic emission maxima: *Amorpha fruticosa* at 480-520 nm, *Robinia pseudoacacia* and

Rubus L. at 420-470 nm, and *Rhamnaceae* at 350-420nm and 570-590nm (Figure 3B, Figure 2). The separation of the pollen samples was most probably due to their specific phenolic composition.

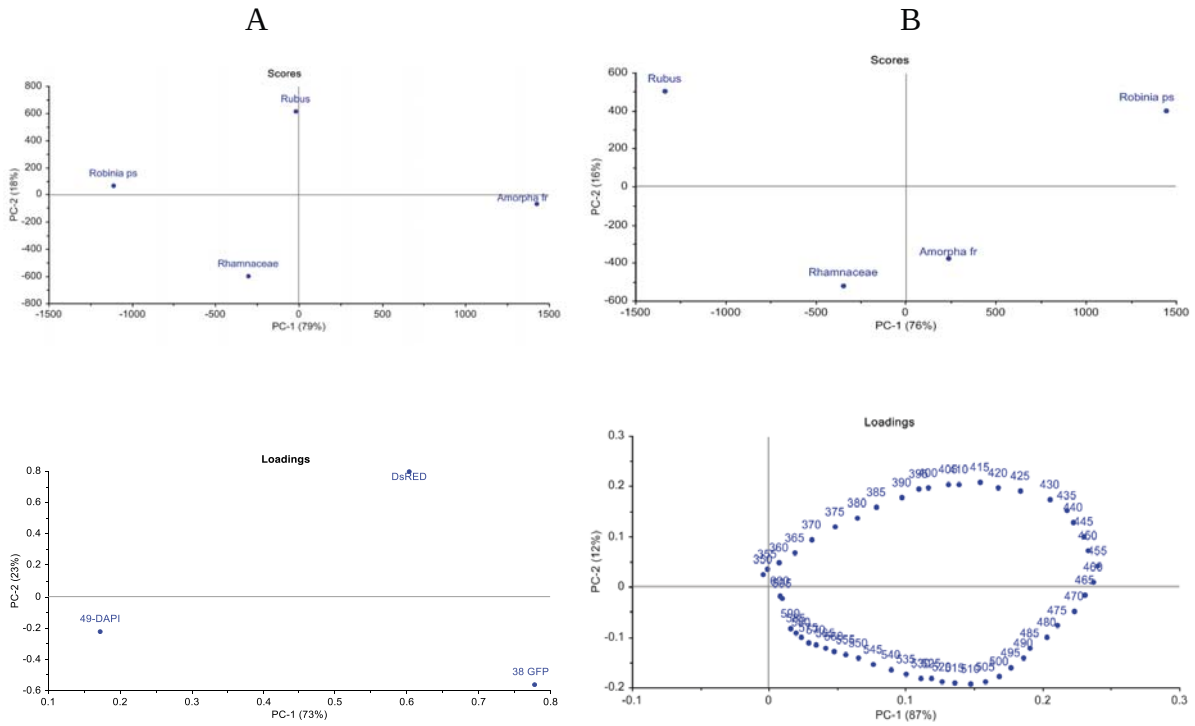


Figure 3. Principal component analysis of : (A) microscopic fluorescence and (B) the fluorescence emission spectra

4. Conclusions

In this pilot research, we showed that pollen autofluorescence varied between the pollen of the different botanical species. Our findings suggest that classification of pollen grains may be obtained based on their fluorescence images.

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