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ANATOMIC AND SPECTROPHOTOMETRIC DIFFERENTIATION OF *DALBERGIA NIGRA* FROM OTHER SPECIES OF ROSEWOOD

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ABSTRACT

Tropical woody plants are characterized by specific anatomic features that help distinguish one plant species from another. Cognation and the resemblance of species within the Genus *Dalbergia* do not enable to specify *Dalbergia nigra* macroscopically as threatened woody plant univocally, within the CITES legislation (Convention on International Trade in Endangered Species of Wild Fauna and Flora). The aim of this scientific work was to define *Dalbergia nigra* on the basis of spectrophotometric differentiation of given species and prove that *Dalbergia nigra* can be clearly differentiated from other species of woody plants by means of reflectance. The research work was also focused on the study of anatomical features of *Dalbergia nigra* and other species of rosewood – especially the distribution of axial parenchyma, structure of vessels and rays of woody plants using the scanning electron microscope.

Keywords: scanning electron microscopy (SEM), wood anatomy, wood colour, spectrophotometric data, *Dalbergia nigra*, *Dalbergia latifolia*, *Machaerium scleroxylon*.

INTRODUCTION

This paper is focused on one of the rarest species, registered in the CITES Convention, which is preferred in the manufacturing of musical instruments and luxury furniture by its sounding qualities and texture. *Dalbergia nigra* had been considered very noble wood of Brazilian primeval forests for centuries. In practice, there is great similarity to the Genus *Machaerium*, which is frequently, and many times, even wilfully confused. The precise distinguishing the rosewood woody plants is therefore the subject of many research studies. Detection and determination of extractive neoflavonoid substance, which was aptly named Dalnigrin, is one of the newest methods for the identification of *D. nigra*. Its presence has been demonstrated in all the analysed samples of the heartwood. The structure of chemical marker has been identified as the neoflavonoid 6-hydroxy-7-methoxy-4-(4-methoxyphenyl)-2H-1-benzopyran-2-one (4′-O-methylmelanettin; Dalnigrin), whereas this has not been detected in any of the reference samples of other Dalbergia species, counting *D. spruceana*, using the LC-MS analysis (KITE *et al*. 2010). The Studies that are using DNA analyses are published to maintain and preserve *Dalbergia* species in Indochina, especially *D. cochinchinensis*. YIN, JIANG *and* YUAN (2016) dealt with morphological characteristics, taxonomy, geographic distribution, conservation class and identification characteristics 10 species of the genus *Dalbergia*.

Methods Qualitative and quantitative wood anatomy, principal components analysis (PCA) and naı¨ve Bayes classification were conducted on 43 specimens of *Dalbergia*, eight *D. nigra* and 35 from six other Latin American species (GASSON *et al*. 2010). The authors concluded that wood anatomy alone cannot distinguish *D. nigra* from all other commercially important *Dalbergia* species.

ESPINOZA *et al*. (2015) proved that it is possible to use Time-of-flight Mass Spectrometer analytical method to determine Dalbergia species.

Dalbergia nigra was integrated into the Annex A of Council Regulation (EC) No. 338/97 of December 9, 1996 since July 20, 1992. Generally, it is not permitted to use the specimens of species that are listed in the Annex A for primarily commercial purposes. Certificate of origin shall be attached to any wood of these species (TAYLOR *et al*. 2012, PAROBEK 2008, PALUŠ and PAROBEK 2010, PAROBEK 2013).

As revealed by several research studies, it is difficult to determine the wood *Dalbergia nigra* on the basis of microscopic features with the assistance of visual and textual identification databases (CITESwoodID created in Germany and CITES Identification manual of tropical woody plants created by Environment Canada).

The aim of work was anatomic study of microstructure using Scanning electron microscopy and spectrophotometric distinguishing *Dalbergia nigra* (Vell.) Allemão ex Benth. from species *Dalbergia latifolia* Roxb. and *Machaerium scleroxylon* Tul.

EXPERIMENTAL PART

Sampling and the preparation of samples for scanning electron microscopy

The samples of *Dalbergia nigra have* been used for microscopic analysis originating from 1974 and *Dalbergia latifolia* from the collection of exotic woody plants of Mr. RNDr. Peter Richtárik (bowmaker.sk); samples of *Machaerium Spp.* manipulated from 2 prisms, originally from Bolivia, in cooperation with JAF Holz Slovakia s.r.o. and Legnami Nicolis s.r.I., Verona (importer of exotic cut timber to Europe) (Fig. 1).

Fig. 1 Manipulation of *Machaerium scleroxylon* prisms to samples for electron microscopy.

Specimens with cross dimensions 10 by 10 mm were manipulated from prisms. Specimens for microscopic analysis of sapwood and heartwood area were further manipulated to specific preparations in cross, radial and tangential section. We have used a sample of heartwood for microscopic analysis, divided into smaller parts, determining specific planes of section (cross, radial and tangential section) using the scanning electron microscope for *Dalbergia nigra* and *Dalbergia latifolia*. The preparation of samples with such a high density required special systematic procedures for softening of wooden structures in order to make an ideal cut using the razor blade. Purity and quality of cuts was checked using the stereo microscope. Subsequently, we stuck each preparation to duraluminum specimen stubs by Polyvinyl acetate adhesive. The preparations were then dried under infrared source, while fixing layer was not deprived of moisture (MAMOŇOVÁ 2013).

24 carat gold plating was made in high vacuum in the device from the company VEB HOCHVAKUUM, Dresden. Microscopic observations were carried out using the SEM Tescan – VEGA TS 5130, at accelerating voltage of 16.7 kV, and the secondary electrons were used for gaining signal (SE detector). Microscopic observations were focused on the major qualitative characteristics in the structure of xylem all of the species studied according to the following characters from the IAWA List (IAWA Committee, 1989):

Wood diffuse porous; Simple perforation plates; Intervessel pits alternate; Vestured pits; Vessel-ray pits with distinct borders; similar to intervessel pits in size and shape throughout the ray cell; Gums and other deposits in heartwood vessels; Fibres with simple to minutely bordered pits; Axial parenchyma diffuse; Axial parenchyma diffuse-in-aggregates; Axial parenchyma paratracheal; Ray width one to three cells; All ray cells procumbent; Ray-cellular composition; Prismatic crystals present.

Measurement of colour spectra of the wood

Samples of sliced veneers originating in Brasil, (*Dalbergia nigra, Machaerium scleroxylon*) and Indonesia (*Dalbergia latifolia*), were used for the spectrophotometric measurements.

The color spectra of the surfaces sliced veneers were measured with the spectrophotometer type MINOLTA of CM 2600d. With aid of „SpectraMagic“ software we contrived the measurement processes as well as working with measured data. To delimitate the sample surface to be measured there was used a standard measuring aperture of 8 mm diameter. Illuminating system was set to a measuring mode including scattering components (SCI). We carried out measurements of wave lengths ranging from 360–740 nm, with resolution 10 nm, when a xenon discharge tube D65 was used as a source of light. The spectrometer can measure also a course of spectrum dependent upon reflected wave lengths (MAMOŇOVÁ 2009). It is measured through “reflectance – reflectiveness” representing an ability of the substance surface to reflect an incident light. It is stated in percentages as a ratio between reflected light (radiation) and an amount of reflected light from a reference surface (a white reference with 100%). Measurements were taken on samples that were not treated with any surface treatment (MC 12%).

Statistical indicators of 160 randomly selected measurements on each measured surface of sample were processed in Microsoft Excel. Number of measurements we determined on the basis of variance ($n \rightarrow 40$) of measured values. Spectrophotometric data obtained were measured at a significance level of $\alpha = 0.05$. It was necessary to take at least 160 measurements for each surface of sample in order to estimate mean for colorimetric data of main unit of veneer samples with an error of 5% and a 95% level of confidence. Number of measurements was investigated for each collecting optical channel in the wavelength range 360–740 nm (for each wavelength with a pointiness of bandwidth of 10nm).

We always compared two files with selected data in order to compare data from spectral analyses. Test statistics of two-sample t-test and homogeneity test were used for testing.

Testing was carried out for each wavelength with a 10 nm bandwidth discretion (40 t-tests), and the results of the testing are shown in Graphs 1–4.

The calculated *t*-statistics were compared with tabulated critical value $t_{\alpha(\nu)}$, where ν is the number of degrees of freedom and the level of statistical significance α 0.001 (see Tables of critical values of Student's t-distribution, KLEIN *et al.* 2002, CHAJDIAK *et al.* 1997).

RESULTS AND DISCUSSION

The results of microscopic analyses of ***Dalbergia nigra*** **(Vell.) Allemão ex Benth.** using the SEM Tescan Vega *Dalbergia nigra* has the largest vessels among all the species studied. The average tangential vessel diameter (IAWA Committee 1989) reaches 291.2 μm (min. 149.1; max. 353.0) μm, and they are arranged solitary (Fig. 2a). There were not observed small-diameter vessels within the lower limit of interval (75–175–250) μm as presented by RICHTER and DALLWITZ 2000 and also WAGENFÜHR 2007, showing tangential vessel diameter interval (90–170–260) μm.

It was diagnosed that *D. nigra* had heterogeneous rays in radial (Fig. 2b) and tangential section (Fig. 2d).

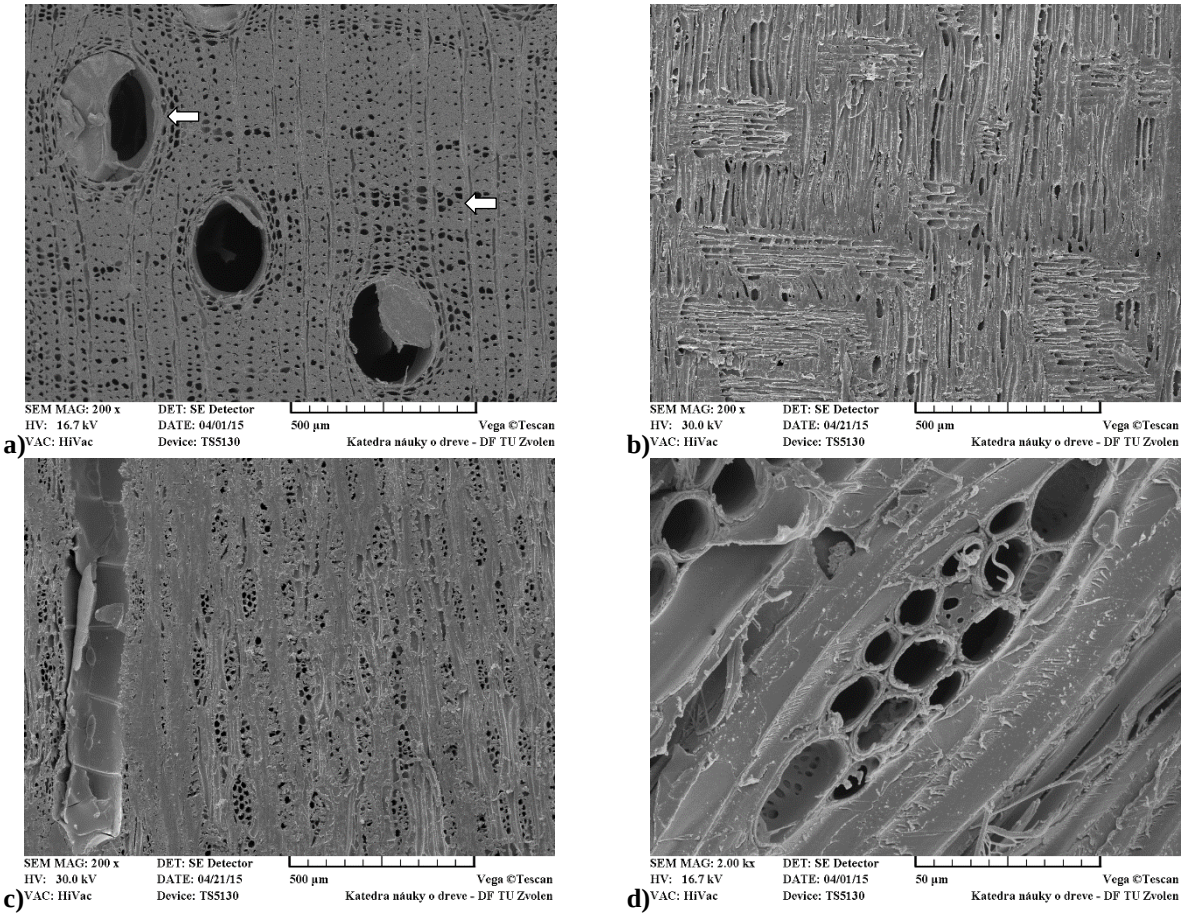


Fig. 2 Anatomic features of Rio palisander wood (*Dalbergia nigra* (Vell.) Allemão ex Benth.). a) Large solitary vessels of elliptical cross-section. Axial parenchyma - vasicentric, round about vessels, apotracheal, streaky - arrows. Thylosis formation in heartwood vessels, cross-section; b) Heterogeneous rays, axial parenchyma, radial section; c) Very symmetric arrangement of 2-seriate and 3-seriate rays; vessel filled with tyloses, tangential section; d) Detail of 3-seriate heterogeneous ray – with one row of upricht cells on the periphery, tangential section.

***Dalbergia latifolia* Roxb.** has smaller vessels, arranged solitary, with the presence of unilateral aliform axial parenchyma as strong diagnostic feature (Fig. 3a). Rays are homogeneous (Fig. 3d).

Anatomic features of sapwood area of ***Machaerium scleroxylon* Tul.** of prism 154 are documented in Figure 4. Despite the fact that it is a species which is especially resistant to rot (WAGENFÜHR 2007), we have identified the presence of hyphae in vessels (Figures 4 a, b, d).

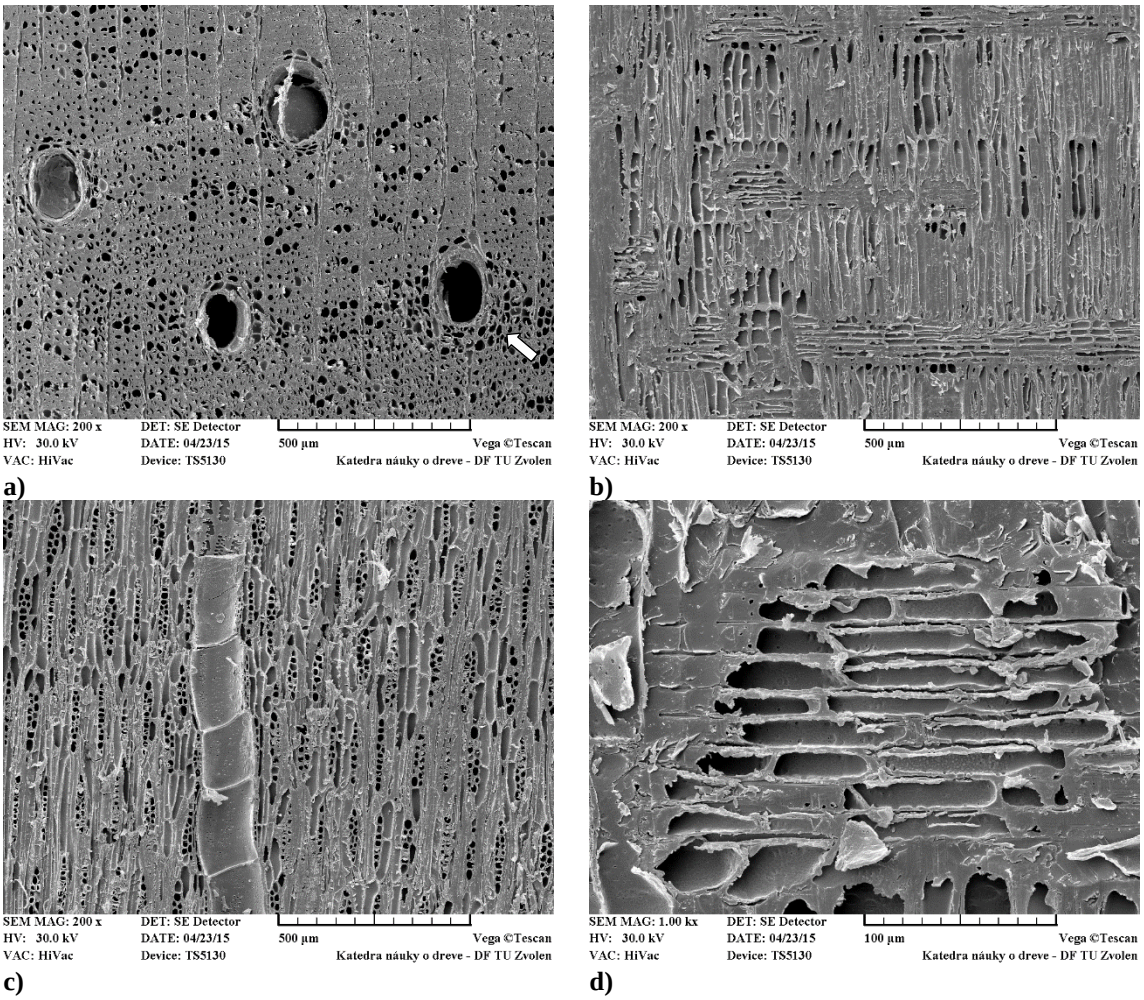


Fig. 3 Anatomic features of East Indian rosewood (*Dalbergia latifolia* Roxb.). a) Small solitary vessels, tylosis formation in heartwood vessels, considerable unilateral axial parenchyma - arrow, cross-section; b) Interconnection between axial parenchyma and ray parenchyma; homogeneous rays, radial section; c) Vessel, apotracheal axial parenchyma; uniseriate to 2-seriate rays, tangential section; d) Detail of homogeneous ray composed of only radial elongated (procumbent) parenchymal cells, radial section.

Anatomic features of heartwood area of ***Machaerium scleroxylon* Tul.** prisms 154 are documented in Fig. 5 and prisms 389 in Fig. 6. In both cases, we have diagnosed the presence of uniseriate rays which determine this specimen univocally (Fig. 5c, Fig. 6c). This species is specific by the presence of pit pairs of vessels (Figures 6c), which are characterized by protrusions in slotted aperture, the so called "vestured pits" (JANSEN *et al*. 2000, IAWA Committee 1989).

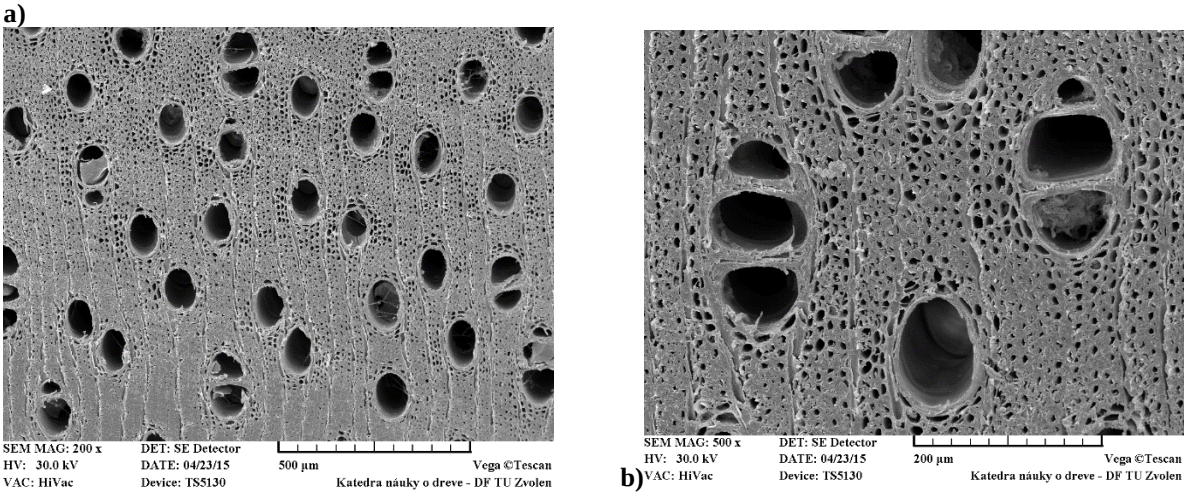


Fig. 4 Anatomic features of sapwood area of Santos palisander (*Machaerium scleroxylon* Tul.) – prism 154. a) Sapwood area locally tylosis formation of vessels (probably as a consequence of pathogenic irritation), cross-section; b) Solitary arrangement of vessels, or in short radial groups.

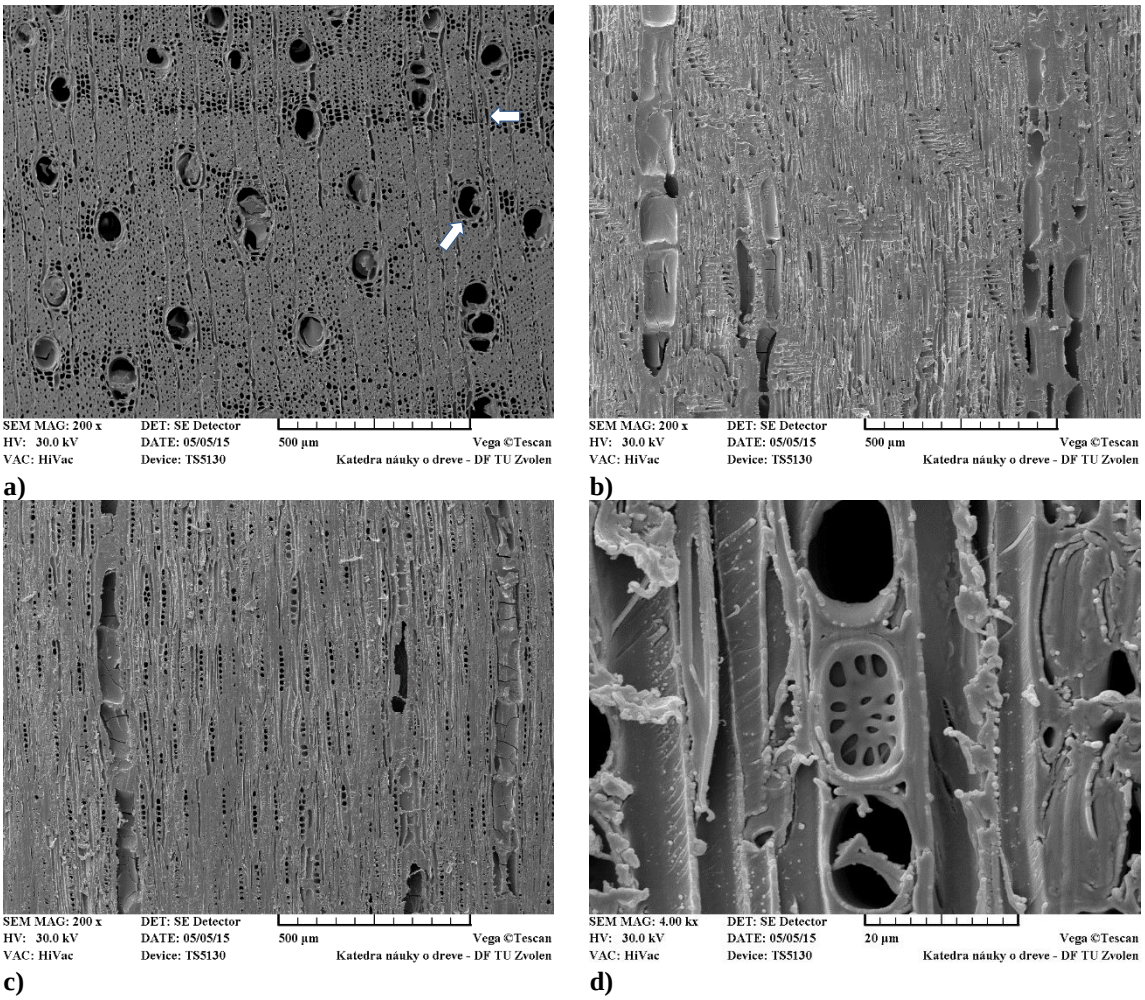


Fig. 5 Anatomic features of heartwood area of Santos palisander (*Machaerium scleroxylon* Tul.) – prism 154. a) Encrustation of vessel lumens, axial parenchyma, paratracheal banded, also vasicentric, thick-walled - arrows, libriform fibres, cross-section; b) Encrustation of vessels, homogeneous rays, radial section; c) Uniseriate rays are predominant, they are arranged in layers, tangential section; d) Detail of membranes of parenchyma of uniseriate ray, tangential section.

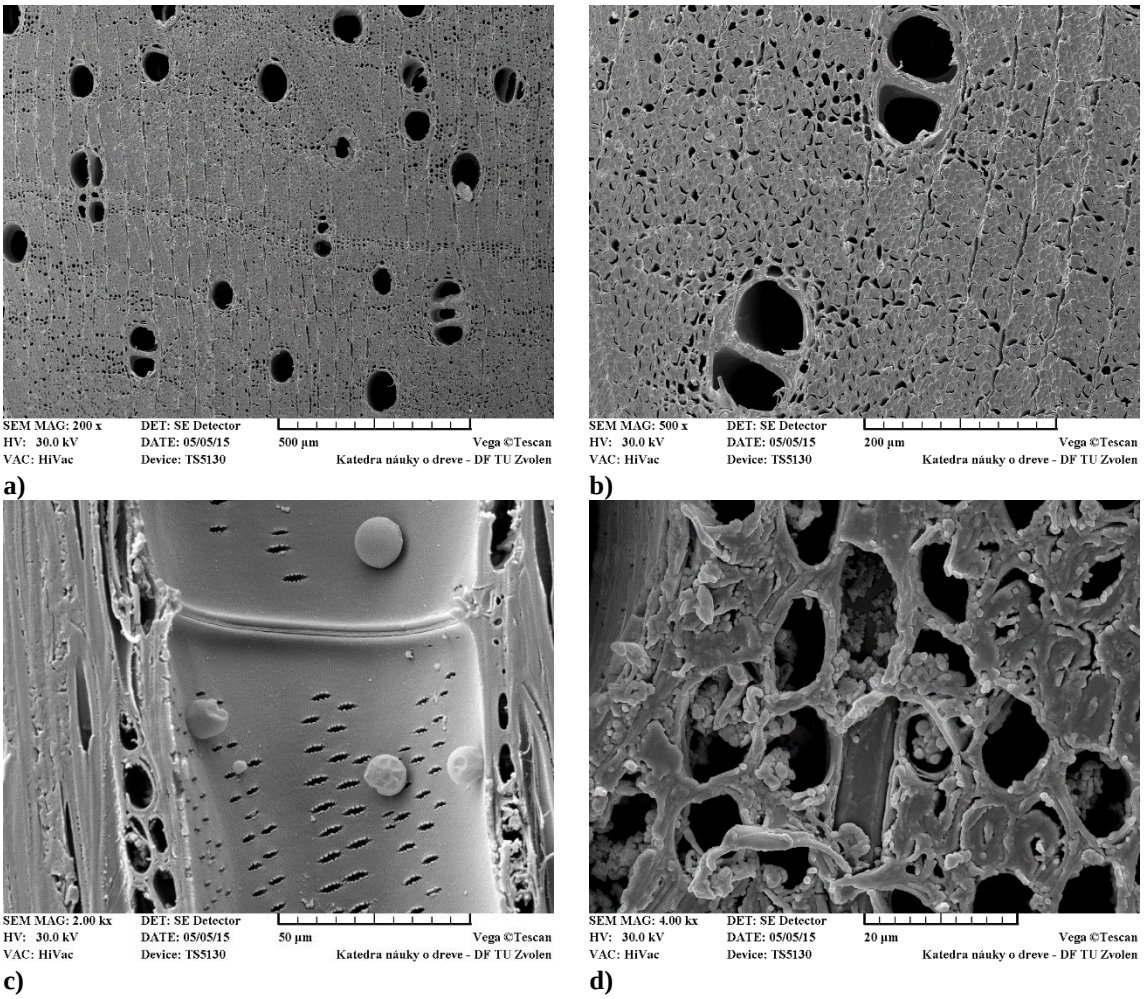


Figure 6 Anatomic features of heartwood area of Santos palisander (*Machaerium scleroxylon* Tul.) – prism 389. a) Solitary arrangement of vessels, or in short radial groups; banded axial parenchyma, cross-section; b) Detail of libriform fibres of tension wood, cross-section; c) Beginning of vessel tylosis formation and the presence of „vestured pits“ on vascullar walls; tangential section; d) Detail of heartwood substances in axial parenchyma, cross-section.

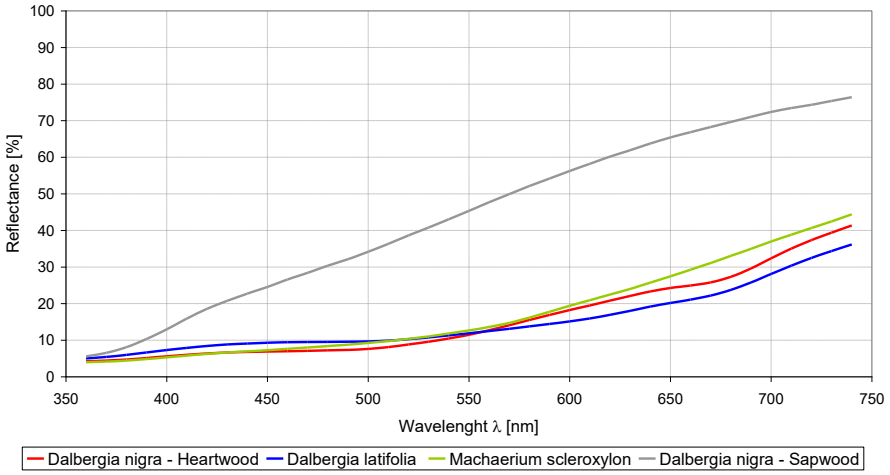
Results of spectrophotometric analyses

In addition to the colour, spectrophotometer is also capable of measuring the course of the spectrum which is dependent on wavelength reflected. It is measured through “reflectance – reflectiveness” representing an ability of the substance surface to reflect an incident light. It is stated in [%] as a ratio between reflected light (radiation) and an amount of reflected light from a reference surface (a white reference standard with 100[%] reflectance).

Within the reflectance it is possible to analyse hue changes due to a paint coat on a wood in whole range of a visible spectrum – in 40 discrete wavelengths of light. The complicatedness of the solution occurs, when we want to differentiate given species on the basis of certain significance level. In our case, when we want to differentiate *Dalbergia nigra* from other woody plants with respect to genera, or species.

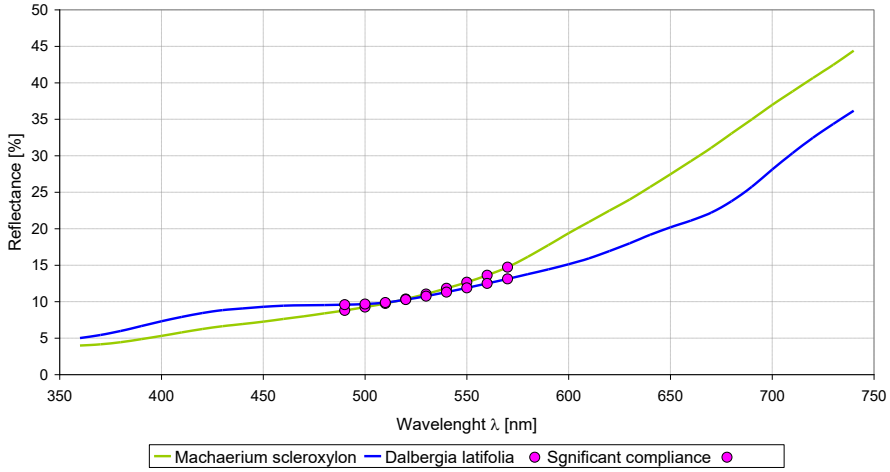
Graphs 1–4 show the course of detected colour spectra of individual examined veneer surfaces of genera *Dalbergia* and *Machaerium*. The final course of colour spectra reveals the known fact that it is very difficult to differentiate these woody plants subjectively. The spectral curves show very similar course which is at first sight significantly different from all the courses of spectral curves, except for the waveform of sapwood part of *Dalbergia nigra*. The sapwood part of investigated species is relatively narrow part of wood, which is,

in addition, rarely used for decorative purposes in the case of *Dalbergia latifolia*. Therefore, we focused on the heartwood part of investigated species in testing and indication of significant compliance in the graphs (Graph 2 and Graph 4).

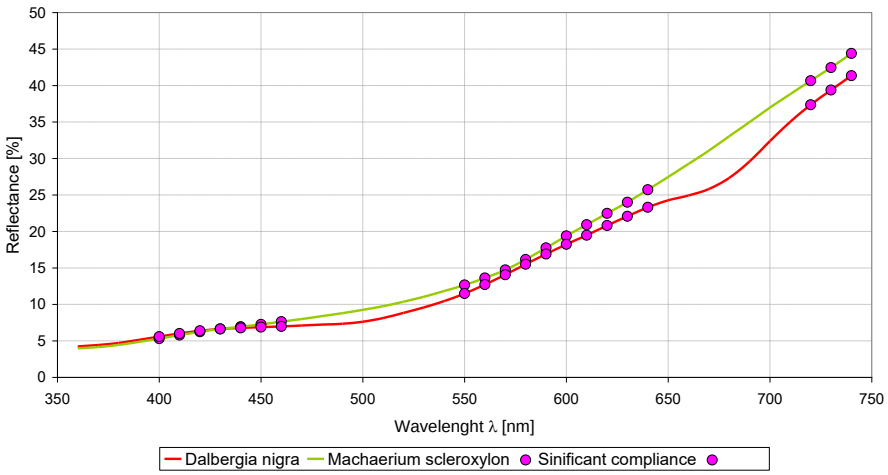


Graph 1 The course of colour spectra in related species *Dalbergia nigra, Dalbergia latifolia and Machaerium scleroxylon.*

We have found out that there was a statistically significant difference between the spectrophotometric data for the veneer surfaces of *Machaerium scleroxylon* and *Dalbergia latifolia* (at $\alpha = 0.001$) within the wavelength interval $\lambda < 360; 480 >$ and $< 530; 740 >$ [nm] (Graph 2). Using the reflectance, we are able to distinguish these two specimens of woody plants on the wavelength ascertained, with a certainty of 99.99%. A significant compliance in reflectance occured within the wavelength interval $\lambda < 490; 570 >$ [nm], in 22,5% of spectrophotometric data measured. There has been a statistically significant difference in 77.5% of course of spectrum between the related species *Machaerium scleroxylon* and *Dalbergia latifolia*.



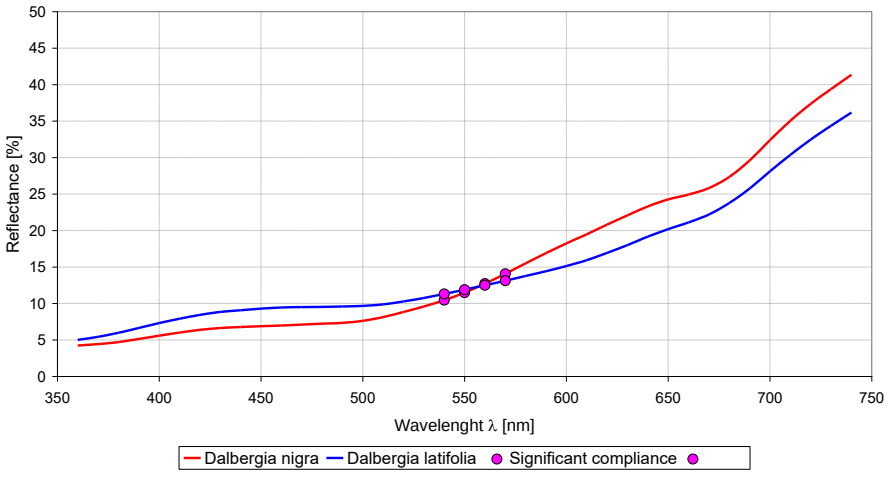
Graph 2 The course of colour spectra in related species *Machaerium scleroxylon* and *Dalbergia latifolia* with indication of significant compliance.



Graph 3 The course of colour spectra in related species *Dalbergia nigra* and *Machaerium scleroxylon* with indication of significant compliance.

As we have already mentioned in the foregoing text, Rio palisander (*Dalbergia nigra* (Vell.) Allemão ex Benth.) can be very difficult differentiated from the species Santos palisander (*Machaerium scleroxylon* Tul.). Just this resemblance is the problem of European and also Global importance. As it has been shown by authors of the most comprehensive quantitative anatomical study for identification of *Dalbergia nigra* GASSON *et al*. (2010), where 43 samples of genus *Dalbergia*, 8 samples *D. nigra* and 35 samples of other Latin American species were analysed, an unequivocal differentiation of *Dalbergia nigra* on the basis of anatomical features (i.e. diameter of vessels, frequency of occurence of solitary vessels, and others), including the PCA analyses, was only in the case of *D. cearensis.*

The significant compliance was also confirmed in the colour spectra of *Dalbergia nigra* and *Machaerium scleroxylon.* It was detected in 50% of the measured spectrophotometric data in discontinuous interval of wavelengths $\lambda < 400; 460 >; < 550; 640 >$ and at very long waves (at infrared frequency) $< 720; 740 >$ [nm]. Clear determinancy of *Dalbergia nigra* with a statistically highly significant difference (at $\alpha = 0.001$) is only possible in certain wavelength intervals (Graph 3).



Graph 4 The course of colour spectra in related species *Dalbergia nigra* and *Dalbergia latifolia* with indication of significant compliance.

Very good results were achieved in the differentiation of *Dalbergia nigra* and *Dalbergia latifolia* using the spectrophotometric data where we found a significant compliance only in 10% of data in the interval of wavelengths $\lambda < 540; 570 >$ [nm].

We have found the wavelength at which we are able to differentiate our investigated species from *Dalbergia nigra, with 99,99 % certainty:*
This are wavelength intervals $\lambda < 360; 390 >$ and $< 650; 710 >$ [nm], i.e. ultra-short and long wave zone.

CONCLUSION

Dalbergia nigra is one of the rarest living woody plants on the planet. 300 years of using the beauty of its wood led to the almost complete decimation in Brazil. This woody plant was put on the „Red List of the International Union for the Conservation of Nature (IUCN)“ in 1998. Despite all efforts to save this rare species, Rio rosewood is reaching the European market with wood. Our aim was to find a way by which we could undoubtedly define *Dalbergia nigra* on the basis of the spectrophotometric differentiation of given species.

We have shown that at a particular wavelengths $\lambda < 360; 390 >$ and $< 650; 710 >$ [nm] is it possible to differentiate *Dalbergia nigra* from the other species of woody plants using the reflectance.

This method requires the creation of a broader database of colorimetric data, so that it can be verified in practice for comparison of samples used for industry.

During our investigation we have found that spectrophotometric data are sensitive to naturally ageing sample of veneer.

Using microscopic analysis, we determined that there is a strong similarity and generic overlapping of features such as size and arrangement of rays (which are 2-seriate and 3-seriate arranged), arrangement of axial parenchyma, which is located in wood of all investigated woody plants (apotracheal as well as paratracheal), and does not create precisely defined arrangement and shapes at cross-section.

Nevertheless, we confirmed the patterns that are characteristic for the particular species. These relations are as follows:

- *Dalbergia nigra*: has the largest vessels among all the species studied, they are arranged solitary. We have shown that it has heterogeneous rays.
- *Dalbergia latifolia*: has smaller vessels that are arranged solitary. Homogeneous rays were proved.
- *Machaerium scleroxylon*: it has the smallest vessels of all investigated species which are arranged in short radial small groups grouped by 2 or 3 vessels. It is unequivocally differentiated by the representation of uniseriate rays and the presence of pit pairs of vessels that are typical by protrusions in slotted aperture, the so called "vestured pits". These have not yet been described in any literature for species *Macherium scleroxylon*.

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