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Image analysis – tool for quantification of histochemical detections of phenolic compounds, lignin and peroxidases in needles of Norway spruce

J. SOUKUPOVÁ and J. ALBRECHTOVÁ[1]

Department of Plant Physiology, Faculty of Science, Charles University, Viničná 5, CZ-12844 Prague 2, Czech Republic

Abstract

Image analysis has become a powerful tool for the quantification of histochemical detections mainly in animal and medical sciences. There is not, however, much information about the accuracy of image analysis quantification of histochemical products and their comparison to biochemical analyses. Our study focused on the quantification of histochemically detected amounts of phenolic compounds, lignin and activity of peroxidases in the two youngest age classes of Norway spruce (*Picea abies* (L.) Karst) needles with the use of image analysis and its comparison with biochemical assays. The image analysis-determined amount of lignin and peroxidase activity was well correlated with its biochemical assay. On the other hand, no optical parameters of the reaction product of the histochemical reaction of phenolics correlated with the biochemical quantification. This proves that results of quantification of histochemical detection with image analysis greatly depend on the character and procedure of the histochemical proof. If it is done in full respect of the conditions of a histochemical reaction it can gain reliable and interesting results. Great advantage of the image analysis approach is quantification of the studied substance in different tissues within single sample as well as among different samples, which in many cases cannot be detected with biochemical tools.

Additional key words: biochemical assay, histochemical tests, *Picea abies*.

Introduction

Histochemical detection provides accurate information about the distribution and accumulation of a studied compound in different cells and tissues, but the quantification of the results of histochemical proofs is not easy. Commonly used semi-quantitative evaluation of the intensity of histochemical reaction can be useful to some extent, however, it does not allow the description of less pronounced differences and is to some extent observer-dependent. Cytophotometrical measurements, which were used in the past to overtake this problems, are not suitable for all kinds of detections and need special, expensive instruments.

Image analysis (IA) has become a very powerful scientific tool for measurements of lengths, area or shape and it replaces the old methods for quantification requiring laborious measurements in the microscope (Riederer *et al.* 1988, Moberg 1999, Soukupová *et al.* 2001). IA also offers the whole range of measurements of optical parameters and colour characteristics of objects suitable for the quantification of histochemical proofs. The image analysis allows obtaining more precise and detailed data, thus enabling better comparison of different tissues, treatments, *etc.* There is, however, a lack of information about the accuracy of IA quantification of histochemical tests in comparison to data obtained using biochemical tools. Therefore, our study tried to find out whether the quantification of the histochemically detected amount of phenolic compounds, lignin and activity of peroxidases in needles of Norway spruce with the use of IA could bring reliable quantitative data comparable to biochemical quantification.

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[1] To whom correspondence should be addressed; fax: (+420) 221951684, e-mail: albrecht@natur.cuni.cz

Materials and methods

Material: Samples of one- and two-year-old fully matured, dormant needles (48 samples in total) were collected from six clonal five-year-old nursery grown Norway spruce (*Picea abies* (L.) Karst) trees in the beginning of January 1999. They were divided into four groups (one for histochemical analysis and three for biochemical analyses), weighed and stored in -70 °C prior to following analyses.

Histochemical methods: The middle part of nine needles was mounted into 5 % gelatin and sectioned with cryo-microtome (*SME Cryotome Shandon Inc.*, Pittsburgh, USA) (thickness 30 mm). Nine needles were analyzed per tree and needle age class. The presence of phenolic compounds was determined using Fast Blue BB Salt (O'Brien and McCully 1981) to give a characteristic reddish-brown reaction product. The treatment of sections with phloroglucinol-HCl reaction (Němec 1962) localized lignin deposited in cell walls. To prevent any bleaching of the reaction product, sections were mounted into 25 % sulfuric acid in glycerol and captured by camera immediately.

The activity of peroxidases was determined after incubation in reaction mixture containing H_2O_2 and dimethylaminobenzidine (Smith and O'Brian 1979). To avoid oxidation during incubation, the reaction mixture was changed several times. The reaction was stopped after 20 min by proper rinsing with 10 mM sodium phosphate buffer (pH 6.5), and needle sections were mounted into 75 % glycerol. Control sections were incubated in a mixture in which hydrogen peroxide was substituted with the same amount of acetate buffer.

Image analysis: Needle sections were captured using an *Olympus BX 50* (Tokyo, Japan) microscope and *Olympus DP 10* digital camera. Images were processed with 16 bits colour computer assisted image analysis (software *Lucia*, *LIM*, Prague, Czech Republic) to obtain optical parameters and colour characteristics described in the manual of the software and common to majority of image analysis softwares. Needle tissues with a red or reddish-brown reaction product, indicating the presence of phenolic compounds located in all tissues, were automatically detected and used for the measurement of optical parameters. Presence of lignin (stained red) and the activity of peroxidases (expressed as the amount of brown reaction product) varied highly among needle tissues and therefore optical parameters of their reaction products were measured separately in an individual needle tissue. To correct for the chloroplast greenness of mesophyll cells, the optical density of the non-stained cytoplasm of control sections was measured and subtracted from the optical density of sample for histochemical proof of activity of peroxidases.

Biochemical analysis: Needles were frozen in liquid nitrogen and homogenized using a motorized pestle and extracted in 10 cm^3 of boiling methanol for one and half hour. The total amount of phenolics was determined by Folin-Ciocalteau reagent (Singleton and Rossi 1965) using gallic acid as a standard.

A pellet of previously homogenized needles was suspended in methanol and cleaned with a series of solvents to isolate cell walls (Soukupova *et al.* 2000). Purified cell walls were weighed and treated as described in Lange *et al.* (1995). Lignin quantity was expressed as change in density at 280 nm.

A parallel sample of needles was homogenized in 10 mM sodium phosphate (pH 6.5) and centrifuged at 4 °C for 20 min at 14 000 *g*. Activity of soluble peroxidase (EC 1.11.1.7) was determined in the supernatant as described by Hrubcová *et al.* (1992). Peroxidase ionically bound to cell walls was extracted from the purified pellet with 1 mM NaCl. The reaction mixture (3 cm^3) containing 13 mM guaiacol, 5 mM H_2O_2, 50 mM Na-phosphate (pH 6.5) and 0.1 cm^3 of the plant extract (Soukupová *et al.* 2000). Oxidation of guaiacol was followed by the increase of absorbance at 470 nm. One unit of peroxidase activity is defined as that activity, which results in an increase of 0.1 absorbance unit per min at 25 °C.

Statistical analysis: If normally distributed, data were analyzed with analysis of variance, differences among treatments were detected using the Tukey-Kramer test and correlation was calculated by Person product moment analysis. The Kruskal-Wallis test, the Kruskal-Wallis Z test and Spearman-Rank correlation analyses were used in the case of other data distribution.

Results

Localization of phenolics, lignin and peroxidases: The needles had typical anatomical structure for coniferous needles (Table 1). The epidermis consisted of a single layer of cells (Fig. 1*B*) often containing phenolic compounds (Fig. 1*K, M*). Guard cells of stomata were lignified (Fig. 1*D*). Sclerenchymous hypodermis was very pronounced in the corners of needles and occasionally missing on needle sides. Lignin was detected mostly in the outer parts of hypodermal cell walls (Fig. 1*B*), while the activity of peroxidases was

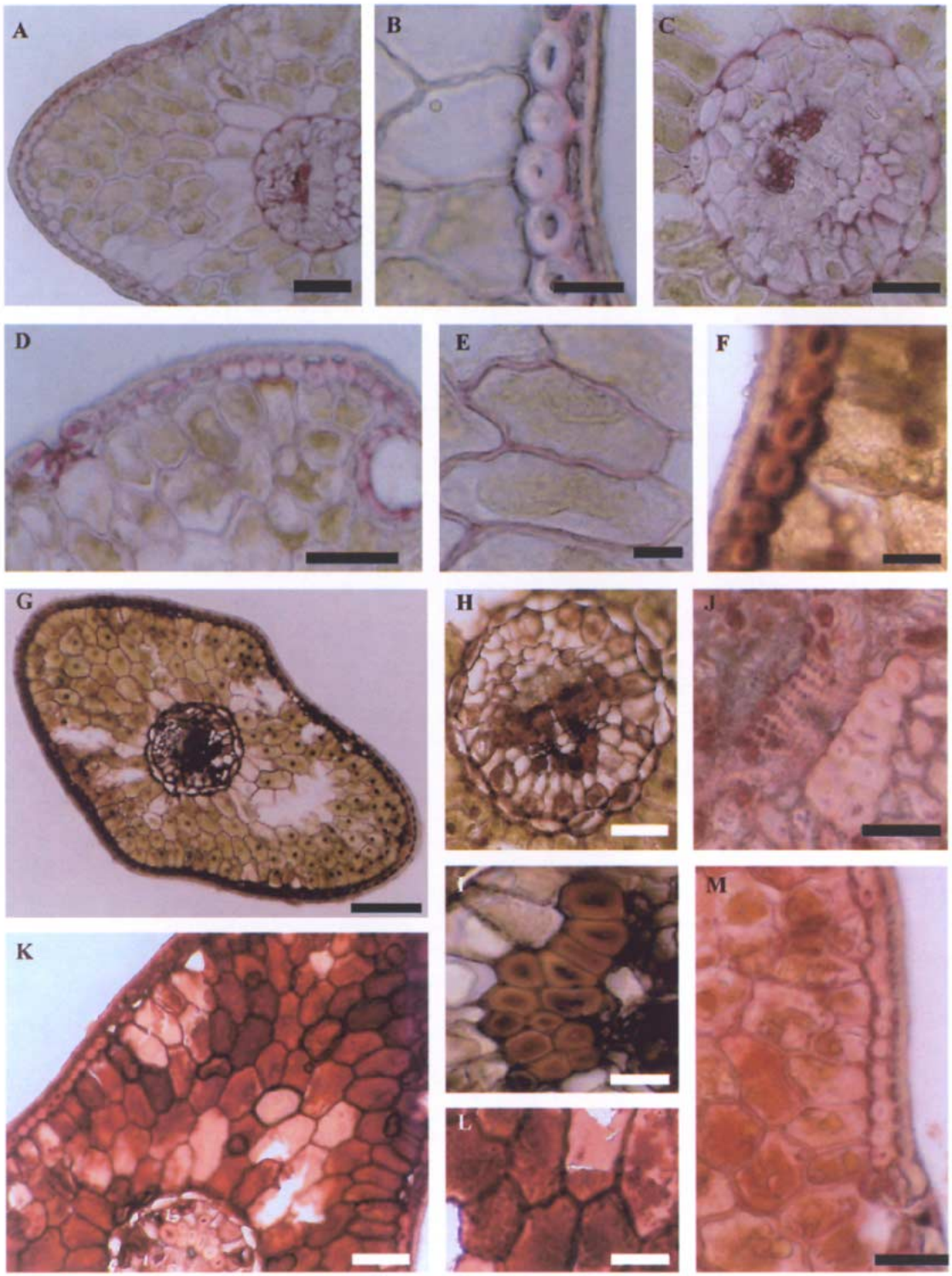
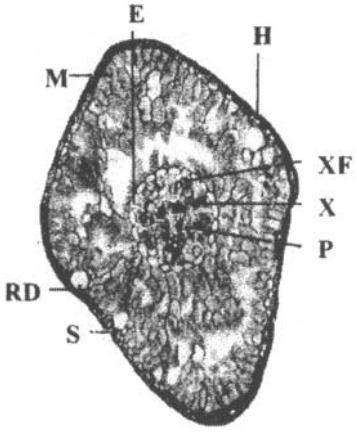


Fig. 1. Localization of lignin, phenolics and peroxidases in needles of Norway spruce. Histochemical detection of lignin was done using phloroglucinol-HCl '(lignin is stained red), phenolics were stained with Fast Blue BB (reddish-brown) and peroxidases (dark brown) were visualised using reaction with hydrogen peroxide and dimethylaminobenzidine. *Bars* represent: 200 μm (*H*), 100 μm (*A, C-E, I, L*), 50 μm (*B, G, K, M*) or 20 μm (*F, J*). *A* - general localization of lignin in needle tissues; *B-E* - detail of lignin localization in epidermis and hypodermis (*B*), endodermis, xylem and sclerenchymatous fibres of a central cylinder (*C*), guard cells of stoma and epithelial cells of resin duct (*D*) and in cell walls of mesophyll cells of second-year needles (*E*);. *F-I* - localization of peroxidase activity in the whole needle (*G*), in cells of hypodermis (*F*), endodermis and central cylinder (*H*) and in phloem and sclerenchymatous fibres (*I*); *J-M* - localization of phenolic compounds in needle tissues (*K*), vascular bundle (*J*), mesophyll cells (*L*), epidermis and endodermis (*M*).

Table 1. Correlation analysis of biochemically detected lignin and peroxidases with optical parameters of reaction products identified by image analysis of peroxidase and lignin histochemical proofs. - no correlation (correlation coefficients lower than ± 0.5, n = 108).

Structure of Norway spruce needle	Lignin			Peroxidases			
					soluble		ionically bounded
	needle tissue	mean op. density	hue typical	needle tissue	mean op. density	hue typical	mean op. density
	stomata (S)	-	r = 0.85	stomata	-	-	-
	hypodermis (H)	-	-	hypodermis	r = 0.72	r = 0.72	-
	epithelial cells of a resin duct (RD)	r = 0.57	-	epithelial cells of a resin duct	-	-	r = 0.94
	mesophyll (M) cell walls	r = 0.70	-	mesophyll cell walls	r = 0.50	-	-
	Casparian bands	r = 0.74	-	cytoplasm of mesophyll cells	-	-	-
	xylem (X)	-	-	nuclei of mesophyll cells	r = 0.53	r = 0.59	-
	xylem fibres (XF)	-	-	endodermis (E)	r = 0.63	-	-
				phloem (P)	r = 0.55	r = 0.57	-
				xylem fibres	-	-	-

associated with their inner parts and cytoplasm (Fig. 1*F*). Resin ducts, if present, were located in two opposite corners of needles. Their epithelial cells had thick and lightly lignified cell walls (Fig. 1*D*) with high peroxidase activity.

Mesophyll cells contained a high amount of chloroplasts (Fig. 1*A*) and big vacuoles with phenolics (Fig. 1*K-M*). Cell walls were usually thin, straight and lightly lignified (Fig. 1*A*), but the presence of irregularly thickened cell walls or clusters of more lignified mesophyll cells (Fig. 1*E*) was noted in the two-year old needles. All mesophyll cell walls were intensively stained with Fast Blue BB indicating a high presence of non-lignin phenolics (Fig. 1*L*). The activity of peroxidases was associated with cell walls, nuclei and cytoplasm (Fig. 1*G*).

Endodermal cells tightly encircling the vascular bundle and transfusion tissue located in the center of a needle were rich in phenolic content (Fig. 1*K, J*). The presence of Casparian bands containing a high amount of lignin was noted in endodermal cell walls of most needles (Fig. 1*C*). Xylem walls were highly lignified (Fig. 1*C*). A certain amount of lignin was found also in the outermost parts of the walls of sclerenchymous fibres (Fig. 1*C*) while activity of peroxidases was detected mostly in their inner parts (Fig. 1*H*). In addition to this, peroxidase activity was detected in the phloem, walls of vessels of protoxylem, transfusion tissue and to some extent also in the functional xylem. (Fig. 1*H, I*). Interestingly, peroxidase activity was higher in the younger part of the phloem located centripetally than in the centrifugal parts of phloem toward the sclerenchymous fibres.

The quantification of histochemical reactions and its comparison to biochemical assessment: According to biochemical measurements, first- and second-year needles contained similar amount of soluble phenolics [56.4 ± 4.42 and 59.4 ± 3.2 mg g^{-1}(d.m.), respectively]. Red stained phenolics were detected in the majority of needle cells (Fig. 1*K*) and their amount was measured in the whole needle, without taking into account tissue-specific location. No correlation was found neither between the amount of phenolics and the mean saturation and optical density (which did not include the optical density and green colour of chloroplasts) nor the ratio of mean red and mean green (which should correct the influence of chloroplast greenness). In agreement with the biochemical quantification of phenolics, no optical parameter showed any significant difference between both needle age classes studied.

The amount of lignin differed greatly within a needle and was measured separately in different needle tissues. Based on the results of image analysis, the highest amount of lignin was found in xylem, Casparian bands and guard cells. The lowest lignification was detected in mesophyll cell walls and hypodermis. According to correlation analysis, the mean optical density correlated greatly with the measured amount of lignin. The best correlation was found in Casparian bands (r = 0.74, Table 1). The overall amount of lignin quantified with image analysis, as well as determined biochemically [first-year needles: 78.5 ± 9.0 A_{280} g^{-1}(d.m. cell walls), second-year needles: 79.8 ± 6.0 A_{280} g^{-1}(d.m. cell walls)], was the same in both needle age classes studied. However, histochemical analysis revealed that mesophyll cell walls and sclerenchymous fibres of two-year old needles contained a significantly higher amount of lignin than one-year old needles (Fig. 2).

Based on results of the biochemical study, ionically

bound peroxidases [first-year needles: 1.46 ± 0.3 μmol g^{-1}(f.m.) min^{-1}, second-year needles: 4.39 ± 0.4 μmol g^{-1}(f.m.) min^{-1}] represented only a small percentage of total peroxidase activity in needles [first-year needles: 24.43 ± 3.52 μmol g^{-1}(f.m.) min^{-1}, second-year needles: 63.28 ± 9.3 μmol g^{-1}(f.m.) min^{-1}]. The total activity of peroxidases was about three-times higher in second-year needles when compared to first-year ones.

The best optical parameters quantifying the peroxidase activity were mean optical density and hue typical (Table 1). The activity of peroxidases was similar in hypodermis, stomata, epithelia cells of a resin duct (if occurred), nuclei of mesophyll cells, endodermis, xylem and sclerenchyma fibres and it was lower in cell walls and cytoplasm of mesophyll cells (Fig. 3). The greatest differences in optical parameters between first- and second-year needles were found in endodermis, phloem, hypodermis and nuclei of mesophyll cells, but it was never as high as in the case of the biochemically-determined activity of peroxidases. The only correlation between the activity of ionically bound peroxidases and the optical parameters of the results of histochemical proofs was found in epithelial cells of resin ducts (r = 0.94).

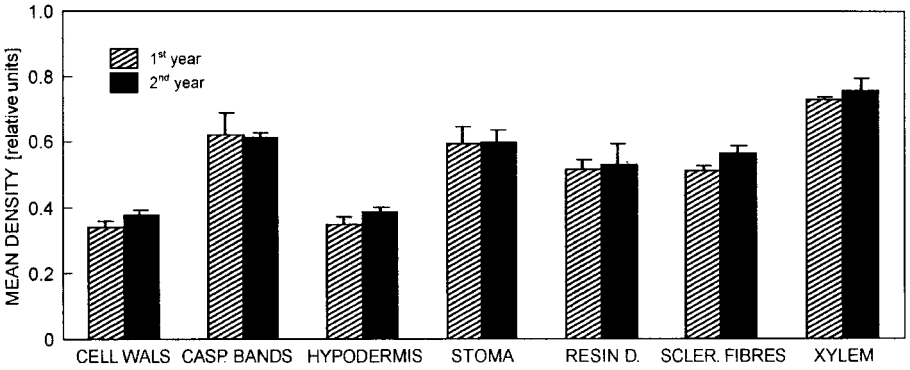


Fig. 2. Mean optical density of the reaction product of histochemical proof of lignin, in different tissues of first- and second-year Norway spruce needles. Mesophyll cell walls ($P \leq 0.05$) and sclerenchymous fibres ($P \leq 0.01$) of second-year needles contained significantly higher amount of lignin than first-year needles. *Bars* represent standard deviation; n = 54.

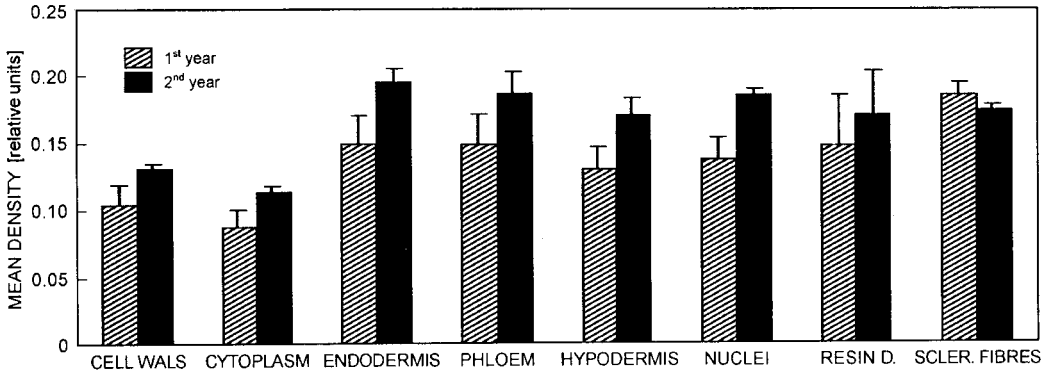


Fig. 3. Average activity of peroxidases in first- and second-year needles of Norway spruce expressed as the mean optical density of reaction product of histochemical reaction measured with image analysis. *Bars* represent standard deviation; n = 54.

Discussion

Image analysis as a tool for the quantification of histochemical or histological results is currently very popular in human and animal histology (Wespes *et al.* 1998, Riera *et al.* 1999), but its use in plant science is still limited. The most commonly used optical parameter for quantification of histochemical detection, which also proved to be the best in our research, is mean optical density (Gardner *et al.* 1996, Fitters *et al.* 1997, Ota *et al.* 1998, Wespes *et al.* 1998). Most of studies assume a good correlation between the intensity of staining and the amount of studied compounds, which does not always have to be true. There are only a few studies, which compare image analysis with biochemical quantification, but these are only in relation to animal material. For example, Fitters *et al.* (1997) correlated successfully the amount of lipids with the optical density of histochemical

staining.

Quantification of histochemical results has several assumptions. It is very important to assure the same thickness of sections. They should be thick enough to obtain staining of greater intensity but also relatively thin to avoid summarization of optical densities of the reaction product in the individual cell layers. It is also important to use the test in which the amount of the reaction product created is proportional to the amount of a studied chemical compound. This assumption is usually true for enzyme detection, because coloured reaction product is created during the reaction with the enzyme. In the case of histochemical proof of the chemical compound presented in a certain tissue (for example lignin), the dye is usually specifically bound to the specific functional group. Whether and to what extent the amount of the reaction product is proportional to the amount of chemical substance depends mostly on its type, molecular structure and the distribution of bounding sites.

Third important assumption for the quantification of histochemical tests is that the reaction product of histochemical reaction does not diffuse from the site of its original location (Pearse 1960, Gahan 1984). Diffusion of the reaction product to surrounding tissues can cause false localization, and diffusion of the reaction product to the incubation media followed by the rinsing out of the reaction product can affect quantitative measurements.

In our study, no optical parameter of phenolics correlated well with the amount of phenolics measured biochemically. Because no leakage of reaction product to media was observed, this fact can be explained either by non-proportionality of the reaction of Fast Blue BB with phenolics, or by differences in biochemical and histochemical methods used. The Folin-Ciocalteau method is a commonly used test for the general quantification of soluble phenolics (Cvikrová *et al.* 1988) but sensitivity of this test is broader than that of biochemical assay used and it can also react with other reductive compounds.

Based on the specificity of the phloroglucinol to coniferylaldehydic groups of guaiacyl lignins, it was possible to conclude that the lignin was of the guaiacyl nature (Norman *et al.* 1990), which is characteristic for Norway spruce (Fengel and Wegner 1989). We proved a high correlation between mean optical density in several needle tissues and the biochemically-quantified amount of lignin. No difference in the amount of lignin detected biochemically between first- and second-year needles was observed. However, using the histochemical approach we found a significant difference in the lignification of mesophyll cell walls and Casparian bands. Differences among lignification of needle mesophyll cell walls of trees of different damage class were found also in study of Soukupová *et al.* (2000) but they were not confirmed with image analysis quantification. This evidence proves not only that the quantification of histochemical test can give reliable results, but also that this approach can reveal new information which would never been found otherwise.

Biochemical analysis proved significant difference in activity of peroxidases between first- and second-year needles. High activity of peroxidase in old or aging tissues was also observed by other authors (Khan and Malhotra 1982, Soukupová *et al.* 2000) and explained in connection with tissue ageing. However, histochemical analysis did not proved any difference in the activity of peroxidases between both needle age classes studied. According to the biochemical test, a high fraction of peroxidases in needle tissues was soluble and therefore, the negligible difference between histochemically determined activities of peroxidases in first- and second-year needles can be explained by diffusion of reaction product to reaction media further promoted by changing of reaction mixture during section incubation. The fact that the lowest peroxidase activity, as well as a lack of correlation with biochemical results, was observed in mesophyll cells, could also be to high extent ascribed to the effect of diffusion because the highest fraction of soluble peroxidases could be expected in cell cytoplasm. Another explanation could be due to methodical approach used including needle freezing and de-freezing before processing, which was inevitable due to a high amount of samples to be processed.

We have proved that the results of the quantification of histochemical results with image analysis greatly depend on the character and procedure of histochemical proof. However, if it is done carefully in full respect of histochemical rules it can gain good, reliable results. In contrast to biochemical detection, the great advantage of the image analysis approach is the description and quantification of studied substances in different tissues, which would not be detected otherwise.

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