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Application of Photoacoustic Spectroscopy in Studies of Environment Contamination Effect on Needles of Scots Pine (*Pinus silvestris* L.)

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Dieback of forests has become a severe problem in Central Europe (Zielski 1997). Different species of trees exhibit a variable sensitivity to contamination. Generally, leafed trees are less sensitive than the conifers, partially since the whole surface exposed to contamination is smaller than that of needles. In addition, leaves, falling off every year, are shorter exposed to pollution than the needles. Scots pine (*Pinus silvestris* L.) belongs to a class of trees which experienced the greatest damages (Huttunen 1978; Palomäki et al. 1996; Hoque and Remus 1999). In the studies of contamination effect on conifers several methods of damage classes are adopted. They are specified by forest research scientists according to the average needle loss of the whole tree, fluorescence spectroscopy, UV, IR, NMR, mass and reflection spectroscopy, microscopy and microscopic image analysis, and histochemical analysis (Hoque and Remus 1999).

In the present paper we describe the application of photoacoustic spectroscopy (one of photothermal spectroscopy versions where a quantity of energy deactivated into heat is evaluated) to quantify the air pollution effect on Scots pine (*Pinus silvestris* L.) needles. The technique of photoacoustics (PA) has been successfully employed to study photosynthetic systems including the direct measurements of the photosynthetic energy storage (ES) (Bultts et al. 1982; Veeranjaneyulu et al. 1991; Tukaj and Szurkowski 1993; Szurkowski and Tukaj 1994; Szurkowski and Tukaj 1995; Szurkowski 1996) and oxygen evolution in intact leaves (Poulet et al. 1983; Kanstad et al. 1983; Malkin and Canaani 1994; Ageev et al. 1998; Malkin 1998; Szurkowski and Kwidzynska 1999). When a sample is exposed to modulated light, a part of the absorbed light energy is emitted in the form of modulated heat (photothermal signal), resulting from the thermal deactivation of pigments. The rest of the energy is dissipated in photochemical processes leading to modulated O_2 emission, and appears as a photobaric signal. Both contributions originate in chloroplasts, from where heat and oxygen diffuse to the cell envelope, and generate acoustic waves in the air phase near the boundary of the cell. The photothermal part of the photoacoustic signal is reduced by a fraction equal to that part of the absorbed energy which is stored by the photosynthetic process as chemical energy. By measuring heat emission in the presence or absence of a nonmodulated saturating light background, the photosynthetic ES is evaluated. One of the advantages of the technique adopted is an opportunity to obtain a depth-profile in the sample studied. By selecting different frequencies of the chopped light, one can obtain information from different depths of a sample. To the best of our knowledge photoacoustic spectra of needles of fir (*Abies alba*) and spruce (*Picea abies*) determined at two frequencies of light modulation were presented in one paper only (Nagel et al. 1987).

MATERIALS AND METHODS

The Scots pine (*Pinus silvestris*) trees selected for studies were from places where the concentration of atmospheric contaminants such as NO_2, SO_2 and flying dust has been measured for several years by the Provincial Inspectorate of Environment Protection in Gdansk. The needles used in the studies were collected in the autumn of 1998 and 1999. The measurements were carried out within two hours after stripping off a tree branch. The places remained after the branches taken were protected against drying. The branches were sampled from the southern side of the pine-tree. The middle part of the needle about 10 - 18 milimeters in length was used for measurements. Three sampling stations were situated in the Tri-city (Gdansk, Sopot, Gdynia) area, and one (Sominy) in a place about 100 km from the agglomeration where the pollution level is very low. For example, differences in the annual impact rates for SO_2 recorded in the studied fields appeared to be very large. The annual SO_2 emission in Gdansk exceeds 20 000 tons whereas the emission in the whole district with the reference station (Sominy) is below 15 tons (data from the Provincial Inspectorate of Environment Protection in Gdansk). Similar differences were noted for emission of other contaminations such as NO_2, CO, light hydrocarbons, dust etc.

The green microalga *Scenedesmus armatus*, isolated from the Gulf of Gdansk of the Baltic Sea (obtained from the Institute of Oceanology, Polish Academy of Sciences, Sopot) was used in this study. A volume of the culture was filtered in such a manner that 10^6 cells were deposited on the sample, covering the filter-ring with a monolayer of cells.

Two supplementary techniques of the PAS signal measurement were used. The first one, photoacoustic spectroscopy, is based on a lab-built PA spectrometer described elsewhere (Tukaj and Szurkowski 1993). Modulated measuring light was produced with a mechanical chopper used with a xenon arc lamp (450 W, OSRAM) and monochromator (SPM-2). Pressure fluctuations in the closed PA cell were detected using a microphone (Type 4146, Brüel & Kjaer). The signal was selected and amplified with a lock-in amplifier (Type 232 B, Unipan). The output was connected to a computer (IBM PC) for further data processing.

The second technique used is based on a laser diode as a light source which allows a precise adjustment of the light beam modulation frequency (*f*) with a very fine step, subsequently performing the sample depth profiling with a high accuracy. In the PAS measurements the sampling depth L decreases with the modulation frequency increase. The sampling depth is given by (Rosencwaig 1980):

$$L = \sqrt{\frac{D}{\pi \cdot f}}, \qquad (1)$$

where D is the sample's thermal diffusivity.

In the studies presented, an experimental set-up based on a lock-in SR 850 amplifier was used, which not only measured the signal but also controlled the operation of the laser diode which was a source of modulated light (Szurkowski and Wartewig 1999). A laser diode (type SDL-73311) emitted modulated light with a wavelength of 680 nm. The modulation frequency varied from 4 to 250 Hz. The adopted modulation frequency range allowed determination of the depth distribution of the signal response for both layers studied. The same modulated light intensity of 25 μmol photons m^{-2} s^{-1} was used in both techniques of photoacoustic signal measurement.

Energy storage was calculated as *(a-b)/a * 100* and expressed as a percentage, where *a* is the photoacoustic signal produced by the modulated light plus the nonmodulated background light of 2500 μmol photons m^{-2} s^{-1}, and *b* is the photoacoustic signal in the modulated light alone. The addition of strong background light saturates photochemical reactions in the sample, increasing the conversion of the absorbed modulated light to heat to nearly 100% and producing a maximum photoacoustic signal proportional to the absorption of the modulated light by the sample. In the presence of the modulated light alone, photochemical reactions are not saturated and a reduced photoacoustic signal is observed as a result of storage of a substantial part of the absorbed light as products of photosynthesis. It should be noted that the ES parameter has been referred to as a photochemical loss. All measurements were performed at room temperature with the measuring light wavelength of 680 nm.

Morphological structure of needles was observed using a Nikon eclipse E800 microscope.

RESULTS AND DISCUSSION

The photoacoustic spectra of an intact Scots pine (*Pinus silvestris*) needles of different ages collected in Sominy and measured at 20 Hz are shown in Figure 1a. Irrespective of the age of the needles (ranging from one to four years) all the photoacoustic spectra demonstrate two maxima at 470 and 680 nm, respectively. The photoacoustic signal rapidly increases on both sides of the visible light range. The general shape of the observed spectra is similar but they differ in the PAS amplitude. The observed shapes of the PA spectra result mainly from an accumulation of light-absorbing, dark pigments (allomelanin) as brown-black bodies in needles (Hoque and Remus 1999). The observed maxima are characteristic of chlorophyll which is the most important pigment taking part in the photosynthesis in green plants. The strong rise below 430 nm and above 720 nm results from the absorption of the chlorophyll-free epidermis and of the cuticula (Nagel et al. 1987).

The examples of photoacoustic spectra for Scots pine needles of different ages collected in Gdynia are given in Figure 1b. The differences observed between the spectra concern also the changes in the spectral shape according to the needles age. For one-year needles even the PA signal is higher by 60% in reference to the sample from Sominy. The signal amplitude variability with the growing age is rather slight. The changes observed in the spectral form concern a continuous disappearance of the maximum at about 680 nm corresponding to the chlorophyll absorption and an increase in the signal amplitude in the range 520-600 nm attributed to the absorption of accessory pigments such as carotenoids. The contribution of the accessory pigments in the PA-signals under optimum photosynthetic conditions appears to be very low (compare the spectral shapes with those in Figure 1a). When the signal is higher in the spectral band studied then the efficiency of energy transfer from the absorbing pigment to the photosynthesis process is lower (Tukaj and Szurkowski 1993).

Energy storage is usually measured at a high modulation frequency (>200 Hz), assuming that there is no O_2 contribution to the signal (Bults et al. 1982). The dependence of ES upon modulation frequency obtained in the measurements for needles Scots pine collected in Sominy and Gdynia is depicted in Figures 2a and 2b, respectively. A form of the dependencies obtained clearly reflects the sampling site location and needles' age. The largest changes were observed for one-year needles collected in Gdynia and Sominy. In both cases considered, ES grows with the light modulation frequency in a similar way up to 150 Hz. For higher frequencies, ES for needles coming from Sominy rapidly increases reaching the value 0.54, whereas for needles collected in Gdynia ES sharply decreases even to a value as low as -0.1 (Figure 2b). The maximum value was taken as a characteristic one of the selected sampling station (Tab.1). For needles collected in Sominy the energy yield of trapping reaches 54% and decreases with the sample's age to 23% for four -year old needles whereas, for needles collected in the municipal areas, the corresponding values are 28-30% for fresh needles, and about 15-17% for the 2-3-year old needles.

Table 1. Photosynthetic energy storage *Pinus silvestris* (annum)

Place of collection	Photosynthetic energy storage [%]			
	1-year-old needles	2-year-old needles	3-year-old needles	4-year-old needles
SOPOT	28 ± 3	15 ± 2		
GDYNIA	30 ± 5	20 ± 2	17 ± 2	
GDAŃSK	29 ± 2	30 ± 2		
SOMINY	54 ± 1	31 ± 2	26 ± 3	23 ± 1

ES for one-year needles from Sominy and Gdynia are presented versus reciprocal square root of the modulation frequency. Such a presentation, after taking the table values for samples (water) thermal diffusivity allows one to relate (via Eq. 1) the modulation frequency to the sample depth where there is the source of the recorded signal (Nagel et al. 1987; Szurkowski and Wartewig 1999). It turned out that the observed variations in ES concern the signal coming from the upper leave layer about 20 μm in thickness. The noticed periodic changes of the signal travelling from deeper layers can be related to a horizontally layered structure of mesophyll of parenchyma cells containing chloroplasts.

In order to determine the structure of the needles, cross sections of green needles from the collecting places were observed in the subsequent years samples. Only cross sections of one-year needles from Scots pine trees (Sominy), indicated the presence of the pigments in epidermis absorbing in the visible range. The excitation of the cross sections of needles with UV light generated not only strong red fluorescence in mesophyll tissues but also in epidermal cell layer. The conversion of UV light in the epidermis into red fluorescence might be useful for photosynthesis by guard cell and for protection against UV damage. For needles collected in Tri-city agglomeration only blue fluorescence of differentiated intensity in the epidermis was observed. A detailed analysis of the role played by epidermis with its pigments in the photosynthesis process, stomatal opening, as well as in protection of plants from UV irradiation can be found in the paper by Hoque et al. (1999).

The structure of needles is rather complex, and cannot be assumed to be uniform (Nagel et al. 1987). In the case of needles, the changes taking place in epidermis and mesophyll cells ought to be interpreted separately. In the case of Scots pine, it is not possible to analyse the photoacoustic signal as a vectorial summation of photothermal and photosynthetic oxygen - evolution contributions in the simple model of Poulet et al. (1983) developed later by Malkin (1998). Such a heterogeneous structure of the samples studied has also the influence on the photoacoustic signal phase change with the modulation frequency (Figure 4). The typical signal phase vs. modulation frequency for young microalgae *Scenedesmus armatus*, with the background light switched on and off is shown in Figure 4a. The studied algae, having transversal dimensions of the order of a few μm, can be assumed as a uniform (homogeneous) sample in the whole range of modulation frequencies. In the case the photothermal signal phase measured with background light is, at all frequencies, lower than the signal measured without background light. The diffusion of heat is faster than that of oxygen in an aqueous medium of the cell and lowers the phase shift of the photoacoustic signal. However, this is not the case for Scots pine (*Pinus silvestris* L.). The phase difference as a function of light modulation frequency for young and old needles is shown in Figure 4b. For higher

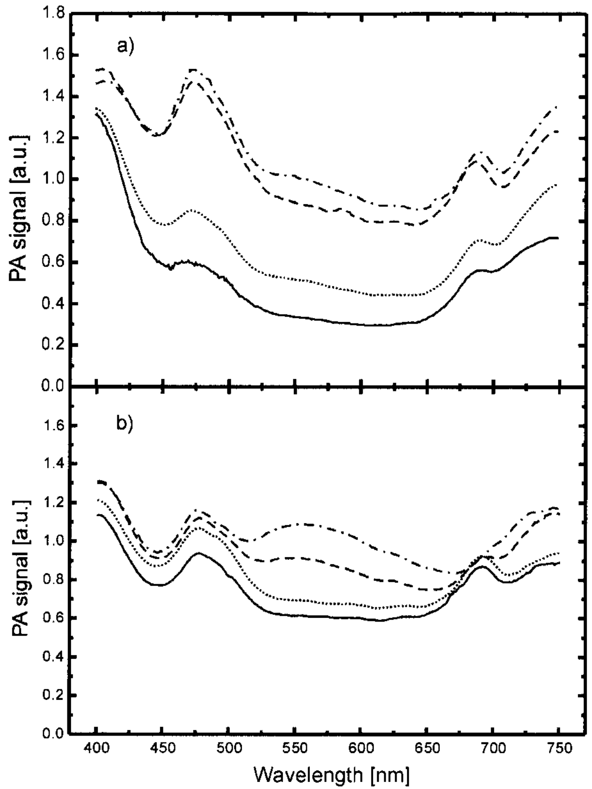

Figure 1. Photoacoustic spectra of needles of (a) healthy - Sominy and (b) a damaged - Gdynia Scots pine (*Pinus silvestris* L.) taken with a modulation frequency of 20 Hz. Spectra are shown for 4 needles ages; (—) - 1 year, (·····) - 2 year, (- -) - 3 year, (– ·) - 4 year

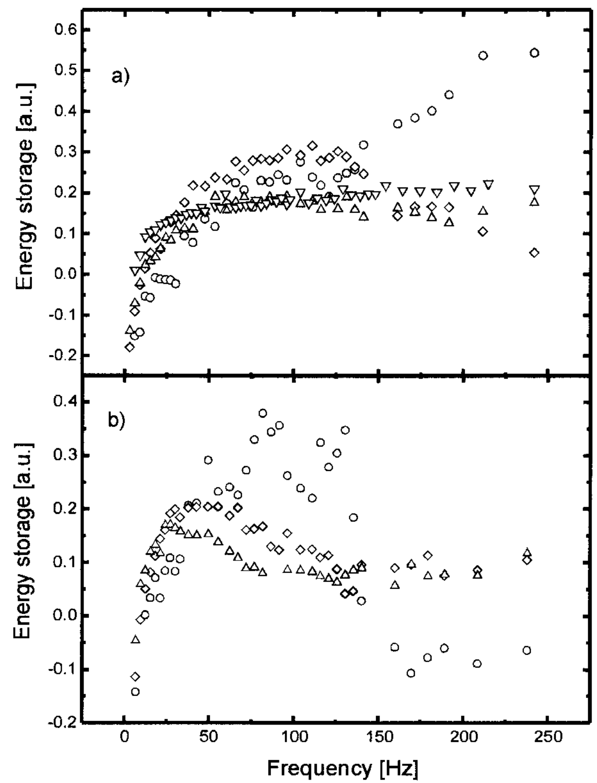

Figure 2. Energy storage of *Pinus silvestris* needles measured at a wavelength of 680 nm versus frequency of modulated measuring light; (a) - Sominy, (b) - Gdynia; (○) - 1 year, (◇) - 2 year, (△) - 3 year, (▽) - 4 year needles.

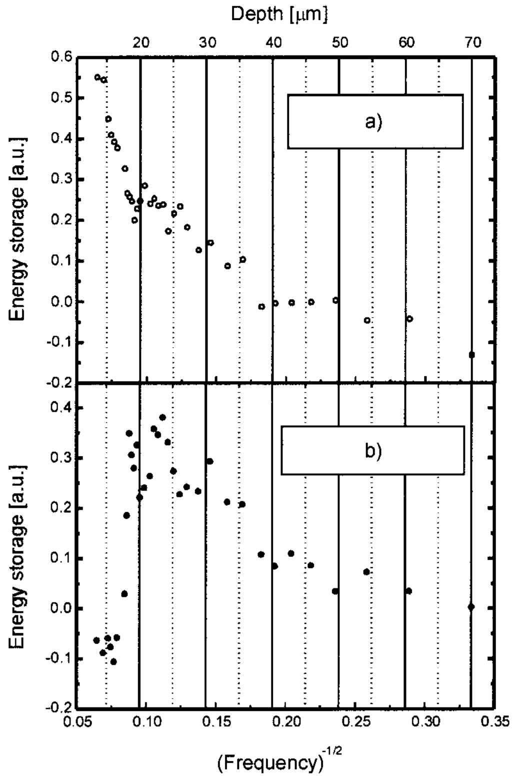

Figure 3. Energy storage of *Pinus silvestris* current year's needles measured at a wavelength of 680 nm versus depth of the sample; (a) - Sominy, (b) - Gdynia.

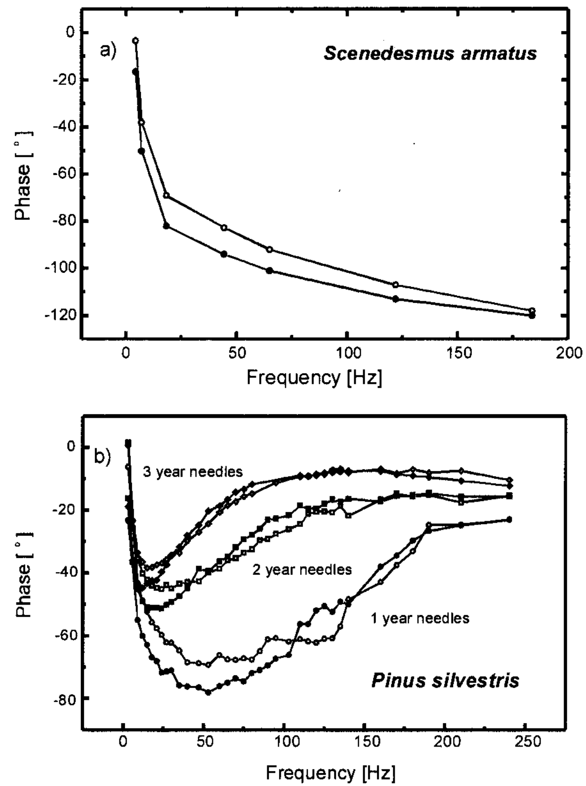

Figure 4. Signal phase vs. modulation frequency dependence, (a) - typical for young microalgae *Scenedesmus armatus*, and (b) - for 1-3 year old *Pinus silvestris* needles, with the background light switched on (open symbols) and off (solid symbols).

modulation frequencies the absolute value of the phase difference decreases because number of light absorbing pigments in epidermis is lower than in mesophyll tissues. It is worth noting that the PA signal phase measured without the background light can be higher or lower than the photothermal signal one. It means that the oxygen evolution signal contributions do not have to be slower than the thermal deactivation time scale. While interpreting the data we must bear in mind that for needles the diffusion resistance is the sum of the boundary layer resistance and the epidermal, cuticular and stomatal diffusion resistances (diffusion resistance is the inverse of conductance).

It has been demonstrated that photoacoustic spectroscopy is one of the measuring techniques allowing the detection of the damage effects to needles and trees. A closer examination of the data obtained for Scots pine (*Pinus silvestris* L.) shows that photoacoustic spectroscopy allows determining several principal parameters in the photosynthetic process, related to the needle structure. The signal amplitude and shape of the spectra demonstrate the age of needles and sampling spatial variability. The signal amplitude increases with the sample age. For needles of trees growing in the Tri-city area with a high level of air pollution, an increase in the photoacoustic signal in the spectral band characteristic of the accessory pigments absorption was observed. Such spectral shape changes resulting from contaminations for other kinds of pine-trees have been already observed by other authors (Nagel et al. 1987).

The photosynthetic energy storage yield (Table 1) is one of the principal parameters characterizing the photosynthetic process. It decreases with the age for all the trees studied. However, despite the age of needles the highest values of ES were observed from 54% (one - year old) to 23% (four-year-old) in the case of samples collected from distant (about 100 km) locations from the Tri-city agglomeration, such as Sominy a place with particularly low air pollution. Analyses of the ES versus modulation frequency showed that a simple model of Poulet et al. (1983) is not applicable to distinguish between the photothermal and photobaric signals. The complex needles structure and epidermis of 20 μm in thickness devoid of chlorophyll create a physical condition when, for high modulation frequencies, the assumption that we are concerned only with the thermal signal is no longer valid. A negative value of ES for one-year-old needles collected in Gdynia and frequencies above 150 Hz (Figures 2b, 3b) indicate that photobaric signal prevails over the photothermal one in the range studied. The dependencies of ES vs. (frequency of modulation)$^{-1/2}$ (Figure 3) allow determination of the epidermis thickness in leaves and to state whether in epidermis the pigments taking part in the photosynthesis process are present.

A heterogeneous structure of needles is responsible for irregular dependencies of the photoacoustic signal phase plotted versus modulation frequency (Figure 4). These dependencies are related to a non-uniform distribution of pigments in the upper layer of leaves and the occurrence of physical conditions when the photobaric signal may diffuse faster than the photothermal one bearing in mind that for a homogenous sample of water the diffusion coefficient of heat is higher by two orders of magnitude than the coefficient of oxygen.

Our results show that photoacoustic spectroscopy as a non-invasive method and independent of light scattering is a valuable tool in forest-decline research for detecting the environmental stress. It appears that many problems concerning the proper interpretation of photoacoustic signal data for leaves of green plants remain to be solved and additional comprehensive measurements can provide new information on signatures of the photosynthesis process in leaves and their structure.

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