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A Low-Frequency Photoacoustic Spectrometer with an RGB Light-Emitting Diode for Evaluating Photosynthetic Activity in Plant Leaves

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Abstract—A photoacoustic spectrometer based on a three-colored light-emitting diode (with peak emission wavelengths of 465, 525, and 640 nm) designed to determine the intensity of photosynthesis in different deep layers of plant leaves is described. The physical properties of the photoacoustic signal were studied at different wavelengths and light modulation frequencies. It was shown that the proposed spectrometer can be employed for quantitative evaluation of heat dissipation and photochemical assimilation of the absorbed light energy in this medium.

Keywords: photoacoustic spectrometer, photochemical reaction, heat dissipation, RGB LED

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INTRODUCTION

The absorption of light by photosynthetic pigments in plant leaves initiates a photochemical reaction involving light-induced charge separation and multistep electron transfer in the reaction center, which ultimately results in CO_2 fixation as glucose and O_2 release [1, 2]. In this process, the energy of the absorbed photons is partially re-emitted by chlorophyll molecules in the form of fluorescence or transformed into heat. Taking into account the competitive relationships between these three channels of relaxation of light-induced excitation, changes observed in one of them can be used to evaluate the other two [3]. In particular, changes in the leaf fluorescence intensity caused by exposure to weak continuous light together with short flashes can be used to determine the quantum efficiency of the primary photochemical reaction and the level of nonphotochemical quenching of the excitation [4, 5]. This principle underlies the functioning of PAM fluorometers, which quantitatively describe the activity of photosynthesis in plants and its changes under different environmental conditions [6].

Direct measurement of heat dissipation when the induced temperature elevation does not exceed 10^{-3} degrees represents a more complex physical task. In plant leaves, which contain numerous pigment molecules that absorb light energy and transfer it into a single photochemical reaction center nearly without losses, the amplitudes of temperature fluctuations are even lower, even at illumination intensities that saturate photosynthesis. Temperature fluctuations on this order of magnitude can be monitored by means of photoacoustic (PA) spectroscopy: changes in the specimen temperature due to absorption of modulated light cause it to expand or contract and thus induce fluctuations of local air pressure [7]. These periodic pressure fluctuations can be recorded as sound using a microphone; to increase the sensitivity of measurements, the specimen is placed into a PA cell, and the signal from the microphone is recorded using a synchronous detector [8].

Along with the described photothermic PA signal, photosynthesizing systems exposed to light with low-frequency modulation can also generate photobaric PA signals: if the period of light modulation is greater than the characteristic reaction time of oxygen release by photosynthesis (several milliseconds), the oxygen release rate will also exhibit periodic oscillations, leading to periodic fluctuations of air pressure [9]. By comparing the amplitudes of PA signals observed in response to modulated metering light and under simultaneous illumination with intense continuous light that saturates photosynthesis, it is possible to identify the photothermic and the photobaric component, which are associated with energy accumulation by photosynthesis and with oxygen release in the plant leave, respectively. This makes the PA method a tool of considerable scientific and practical significance [10].

Light absorption in plant leaves depends significantly on wavelength: the absorption is strong in the blue ($\lambda < 480$ nm) and red ($\lambda > 630$ nm) range, but rel-

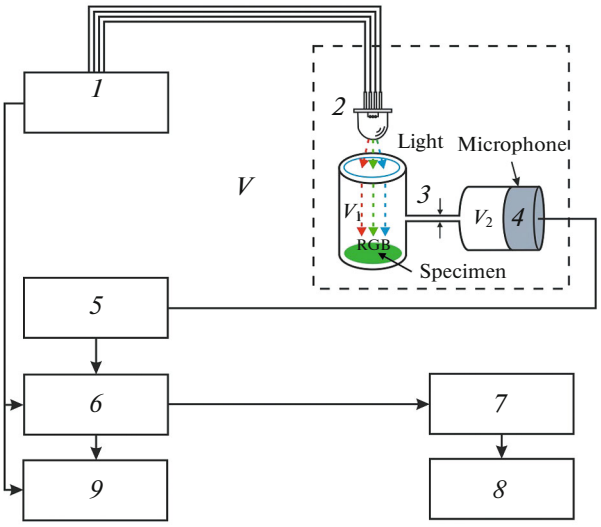

Fig. 1. Diagram of PA spectrometer: (*1*) power supply; (*2*) RGB LED; (*3*) PA cell; (*4*) MK-4 microphone; (*5*) U2-8 preamplifier; (*6*) UPI-2 synchronous detector; (*7*) USB DI16-4 digital input module; (*8*) computer; (*9*) C1-70 oscilloscope.

atively weak in the green range (500–550 nm); as a result, the depth of penetration and the cross-sectional profiles of induced photosynthetic activity differ for radiation of different wavelengths [11]. On the other hand, altering the frequency of light modulation changes the thickness of the medium that generates the acoustic signal, which is employed in PA spectroscopy [12]. Taken together, these two phenomena make it possible to investigate the intensity of photosynthesis depending on the thickness and structural features of leaves that belong to plants with different genotypes. Moreover, a number of serious physical limitations that arise when traditional sources of white light (e.g., incandescence lamps) are used can be overcome by employing new-generation multicolored light-emitting diodes (LEDs) [13] to resolve a narrow spectral band (e.g., the multibeam measuring mode under saturating illumination) [14]. PA methods also provide important advantages for investigating the absorption spectra of optically heterogeneous media and nanostructured materials [15].

In the present work, we describe a PA spectrometer based on an RGB (red, green, blue) LED. The power and the temporal characteristics can be regulated independently for each beam. The proposed spectrometer was applied to investigate the efficiency of energy transformation by photosynthesis in leaves in vivo by simultaneously illuminating specimens with two light beams. The use of an RGB LED as a light source ensured that the optical pathways and beam profiles of light with different wavelengths were identical, whereas the energy requirements on the light source could be relaxed considerably. The possibility of employing the proposed spectrometer for quantitative evaluation of photosynthesis and its fall due to leaf senescence or dehydration has been demonstrated.

EXPERIMENTAL DEVICE AND METHODS

A diagram of the PA spectrometer is shown in Fig. 1. Leave cuttings obtained from white mulberry (*Morus alba*) plants grown in a sunny habitat and from *Spathiphyllum Schott.*, an indoor house plant with low tolerance to intense illumination, were placed in a duralumin PA cell made as a Helmholtz resonator composed of two chambers (for the specimen and and the microphone) [16] with free volumes of $V_1 = 400$ and $V_2 = 150$ mm^3 connected by an air channel 1 mm in diameter. This unit exhibits a high acoustic sensitivity even if the light modulation frequency differs considerably from its resonance frequency [17].

An ARL-5213RGBC/4C LED (Arlight, United States) served as the light source; it comprises three independent emitters: red ($\lambda_R = 640$ nm, AlGaInP), green ($\lambda_G = 525$ nm, InGaN), and blue ($\lambda_B = 465$ nm, InGaN). Depending on the power, the spectral width of each channel was 15–25 nm, and the maximal light power was 50 mW. Owing to the close positioning of the three emitters and the optimal design of the forming optics, all three light beams had identical output profiles with a cross-sectional diameter of ~5 mm and an angular divergence $2\theta = 30°$. So that each emitter can function independently, a three-channel power supply unit was developed, for rigorously sinusoidal modulation of the light intensity of each beam in the 5–500 Hz frequency range or to maintain it at a constant level.

Thus, one of three beams was used as a source of weak modulated light, while one of the others served to saturate photosynthesis. Importantly, all emitters were located in a single RGB LED base mounted directly on the chamber of the PA cell. In some experiments, an optical fiber bundle was used to conduct light in the PA chamber. In all experiments, the intensities of saturating and weak modulated light on the leaf surface in the PA cell were maintained at 350 and 35 W/m^2, respectively. A low-noise MK-4 microphone was used as a sound receiver; the signal was amplified with a U2-8 preamplifier and measured with a UPI-2 synchronous detector; it was also converted using a USB DI16-4 digital input module (Japan) and saved to a computer. The phase shift between the modulated light and the PA signal was monitored using a two-channel C1-70 oscilloscope. To discriminate between the photothermal and the photobaric PA signal components, the latter was suppressed by exposing the leaf to saturating light or by appropriately adjusting the recording phase in the synchronous detector [10, 18].

To investigate the nature of the decrease in photosynthesis during leaf senescence, the contribution of the photobaric component to the total PA signal was

evaluated in autumn, in leaves with different degrees of yellow discoloration, which was assessed by their reflection spectra [19] recorded with an HR2000 spectrometer (Ocean Optics B.V., Netherlands). Simultaneously, the quantum efficiency of the photochemical reaction [20] was determined in the same leaves using a MINI-PAM fluorometer (Germany). Water content in leaves was measured by weighing them immediately after separation from the mother plant and after complete drying.

RESULTS AND DISCUSSION

The study was performed using leaves of white mulberry plants grown in open terrain; the optical properties of these leaves are uniform over their surface, but the cells of the upper and lower layer differ clearly in spatial organization and chlorophyll content [21]. Figure 2a shows the cross-sectional structure of such a leaf. Depending on illuminance during the growth period, the leaf thickness ranged from 128 to 185 μm.

In the cross section, several layers of cells with photosynthetic activity lie between two thin surface layers of nearly transparent epidermal cells. Those cells that are closer to the upper side facing the light contain more chloroplasts, and there is little air space between them. In contrast, the lower layer is composed of cells with fewer chloroplasts, but features more intracellular air space [22]. The bottom panel (Fig. 2b) shows the absorption spectrum of the leaf and the RGB LED emission bands. Blue and red light are strongly absorbed by the top layer cells and hardly reach the bottom layer (in these spectral bands, light transmission by the leaf constitutes 4.5 and 8.5%, respectively), while green light penetrates to the lowermost cell layer, exhibiting transmission of over 21%.

Thus, blue and red light induce photosynthesis mainly in the upper layer of the leaf, which is located closer to the PA-recording system. At the same time, green light acts more uniformly across the leaf, including the lower layer where gas exchange is facilitated due to substantial extracellular air space. These facts imply two consequences: first, the photothermic PA signal induced by blue or red light should have a higher amplitude than the signal induced by green light of the same intensity, and second, since green light retains considerable intensity in the lower part of the cross section, it is expected to induce a stronger photobaric PA signal.

Table 1 shows the amplitudes of the PA signal induced in a leaf of white mulberry exposed to blue, red, and green beams of an RGB LED with low- and high-frequency modulation (6 and 200 Hz, respectively).

The data show that the signals caused by exposure to blue or red light had a nearly three times higher amplitude than those induced by green light and decreased much more dramatically with increasing frequency. The latter observation may be explained by

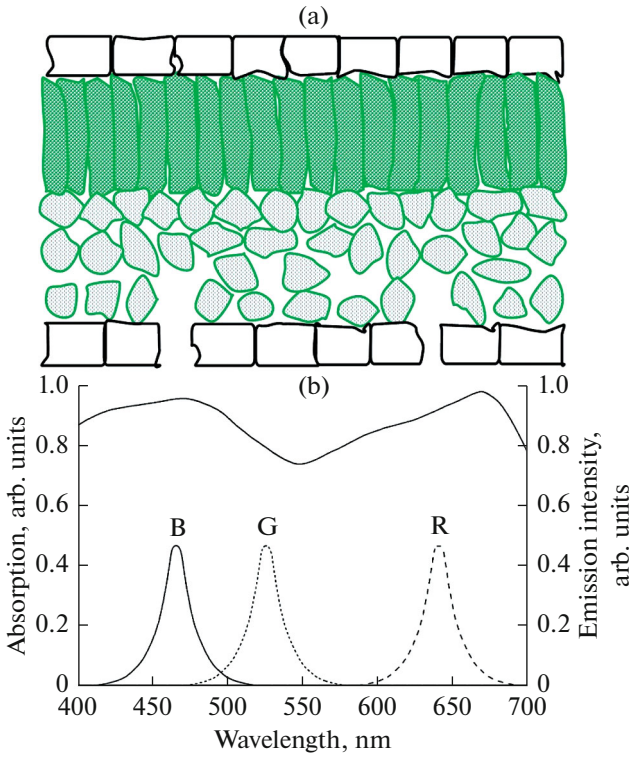

Fig. 2. (a) Cross-sectional structure of a plant leaf; (b) Spectra of light absorption by a plant leaf and bands of light emission by an RGB LED.

the fact that the photothermic PA signal decreases exponentially with depth of the generating site, and the characteristic distance at which the signal drops e times (thermal diffusion length) depends on frequency: $\mu_{pt} = (D_{pt}/\pi f)^{1/2}$, where $D_{pt} = k\rho C_p$ is thermal diffusivity (k is thermal conductivity, ρ is density, C_p is specific heat capacity at constant pressure) and f is the frequency of light modulation [9]. The experimental data on PA signal amplitudes were consistent with this model of signal generation: acoustic waves generated in the lower layer dissipate on a distance of μ_{PT} and may fail to reach the upper surface of the leaf. Taking into account that a white mulberry leaf contains up to 75% water [23] and using the known thermophysical properties of water with the above formulas, it can be calculated that, for $f = 6$ Hz, $\mu_{pt} = 78$ μm, and for $f = 200$ Hz, $\mu_{pt} = 14$ μm. The fact that the frequency

Table 1. Amplitudes of PA signal induced in white mulberry leaves at different wavelengths and light modulation frequencies

Frequency	Amplitude of PA signal		
	$\lambda_R = 640$ nm	$\lambda_G = 525$ nm	$\lambda_B = 465$ nm
6 Hz	8.9	3.0	9.3
200 Hz	3.67	1.7	3.72

Table 2. Changes in PA signal (per cent) at different wavelengths and modulation frequencies caused by additional illumination of white mulberry leaves with saturating light

Frequency	Amplitude of the PA signal		
	$\lambda_R = 640$ nm	$\lambda_G = 525$ nm	$\lambda_B = 465$ nm
6 Hz	–46%	–79%	–43%
200 Hz	+3.8%	+7%	+3.2%

change caused a decrease in the amplitudes of the PA signal that was disproportional to the decrease in thermal diffusion length (Table 1) can be explained with an exponential decrease of light with depth, which is characterized with an exponent of L_{EA} (effective absorption path length). The L_{EA} values calculated for blue, green, and red light based on the absolute absorption values (Fig. 2) and the thickness of the leaf (138 μm in the specimen studied) were 45, 90, and 56 μm respectively. According to the above formulas, at 6 Hz the thickness of the leaf layer that generates a photothermic PA signal in response to blue or red light slightly exceeds the effective absorption path length ($\mu_{pt} \geq L_{EA}$), but at 200 Hz , it is more than three times smaller ($\mu_{pt} = 0.31 L_{EA}$). On the other hand, for green light, $\mu_{pt} \leq L_{EA}$ even at 6 Hz and becomes even smaller at 200 Hz ($\mu_{pt} = 0.16\ L_{EA}$). Changes in the PA signal amplitudes (per cent) for the three wavelengths and the modulation frequencies of 6 and 200 Hz, caused by exposure to additional saturating light are shown in Table 2 [18].

Although previous publications discussed the possibility that the photobaric PA signal in plant leaves may be associated with different gas exchange processes in photosynthesis, it is currently assumed proved that it is mainly due to oxygen release [24]. Data shown in Table 2 suggest that the share of the photobaric component in the PA signal induced by green light is significantly larger than in the signals induced by blue or red light. For low-frequency modulation (6 Hz), exposure to additional saturating light results in a decrease of the total PA signal, because the photobaric component of the PA signal, which is suppressed by this exposure, dominates over the slightly stimulated photothermic component; i.e., the total effect is negative [25]. With increasing modulation frequency, the amplitude of the photobaric PA signal decreases exponentially with a coefficient $\mu_{pb} = (D_{OD}/\pi f)^{1/2}$, where D_{OD} is the coefficient of oxygen diffusion in water. At a low modulation frequency (6 Hz), μ_{pb} is approximately 2 μm; i.e., it has the same order of magnitude as the distance from a chloroplast, as a site of photosynthesis, to the nearest extracellular air space [26]. Taking into account that extracellular air space is predominantly present in the lower layers of the leaf, it seems obvious that green light, which penetrates more deeply, can induce the release of higher amounts of oxygen than blue or red light. However, at high modulation frequencies (200 Hz), the photobaric PA response caused by oxygen release in photosynthesis with its characteristic time of several milliseconds is overshadowed by the nearly instantaneous photothermic PA signal, and the total effect of saturating light on the PA signal amplitude is positive [25].

Thus, by comparing the amplitudes of the PA signals induced in the presence and absence of saturating light, it is possible to assess the intensities of energy accumulation and oxygen release based on the values of the photothermic and photobaric components of the PA signal. These parameters can be employed to monitor the efficiency of photosynthesis in plants with different genotypes and under different environmental conditions. Below, we describe application of the PA spectrometer to evaluate changes in the intensity of photosynthesis caused by leaf senescence.

It is known that leaf senescence involves gradual degradation of pigments and protein complexes that constitute the photosynthesizing apparatus, which is accompanied by significant changes in the reflection spectrum and the rate of photosynthesis [27, 28]. For instance, chlorophyll degradation, which generally precedes the degradation of carotenoids, can be observed as characteristic leaf yellowing [29]. Figure 3 shows the corresponding changes in the reflection spectra of white mulberry leaves. The spectrum of a green leaf (curve *1*) features a carotenoid band at 450–550 nm, as well as an intense chlorophyll band at 650–730 nm, which becomes lower in the spectrum of a yellowing leaf (curve *2*). Figure 4 shows the kinetics of changes in the PA response in these leaves exposed to additional illumination with saturating light. In a green leaf with fairly intense photosynthesis, the photobaric component accounts for 78% of the total PA signal, whereas in a yellowing leaf, its share drops to 37%. These results are qualitatively consistent with the data on quantum efficiency of the photochemical reaction in the same leaves (0.83 and 0.58, respectively) as measured simultaneously using a PAM fluorometer [6]. The discrepancy between the data obtained by PA spectroscopy and PAM fluorometry in a yellowing leaf is probably because a decrease in activity of the oxygen-releasing protein complex and of the electron transport complex of photosystem II occurs at different stages of general degradation of the photosynthetic apparatus [27].

Plant dehydration, an important stress factor of practical significance, suppresses photosynthesis by decreasing the rates of CO_2 fixation and the Hill reaction [30]. There exist several mechanisms characterized by different kinetics that affect the rate of photosynthesis in dehydrated leaves [5, 31]. Strong dehydration of a leaf can partially destroy photosynthetic membranes and thus disrupt the connection between the functional units involved in photosynthesis [32]. This also decreases tolerance to photoinhibition [33].

To elucidate the nature of these changes, we investigated the kinetics of the photobaric PA signal in

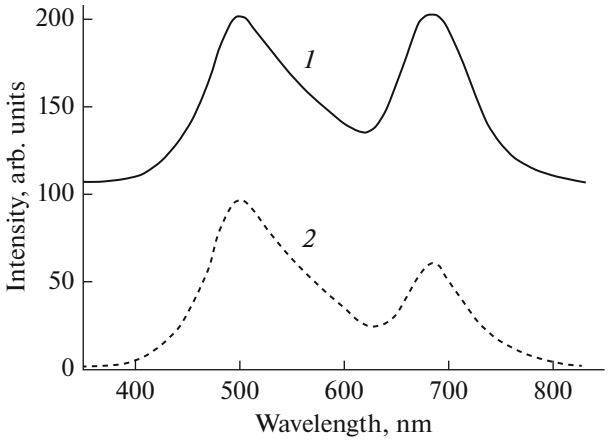

Fig. 3. Reflection spectra of green (*1*) and yellow (*2*) leaves of white mulberry.

leaves of spathiphyllum, an indoor plant with poor resistance to dehydration and strong illumination [34]. The PA signal was measured in leaves at different moments after their separation from the plant, i.e., in specimens with different water contents η. The decrease in photosynthesis was monitored in a specimen exposed to continuous irradiation with both blue and red light with a total intensity of 350 W/m^2. In a freshly detached leaf with η = 74%, the amplitude of the photobaric PA signal was 0.93; at η = 59%, it was 0.35; and at η = 51%, it was only 0.16 arb. units (Fig. 5). Furthermore, irrespective of water content in the leaf, the characteristic fall time was 4.5 min. With further dehydration, the signal decreased to the noise level and could not be analyzed quantitatively. Thus, the two stress factors (dehydration and high-intensity illumination) had a synergistic effect on the rate of photosynthesis.

The rate of photosynthesis, as expressed in the amplitude of the photobaric PA signal, decreased with decreasing η, but also continued to fall under further illumination with intense constant light. This phenomenon could be considered as photoinhibition of photosynthesis; however, the observed fall time of ~5 min was significantly shorter than the known characteristic times of this process [35]. In a previous work [5], we identified three components of nonphotochemical quenching of excitation in plant leaves with characteristic times of 1.1, 8, and 35 min; they were attributed to accumulation of energy in photosynthetic membranes, to state II–state I transition, and to photoinhibition, respectively. Presumably, the same mechanisms could be involved in the decrease in the photobaric PA signal in the current experiment. Since the first two mechanisms are nondissipative, the changes they induce should be reproducible. However, our experiments showed that even after the specimens were subjected to dark adaptation for 10–20 min, the fall curves were only partially reproducible, and in leaves where water content dropped below 50%, the signal

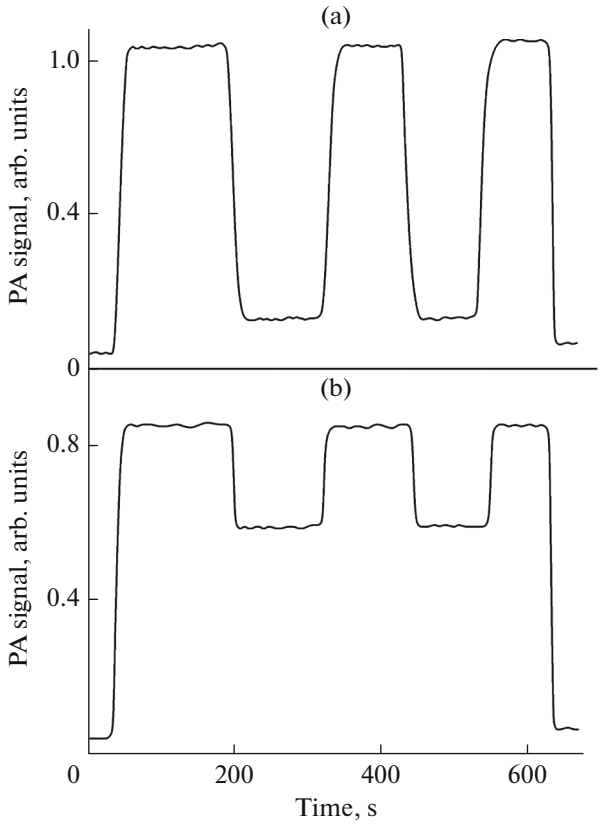

Fig. 4. Kinetics of changes in PA signals induced in green (a) and yellow (b) leaves of white mulberry exposed to additional illumination with saturating light.

was indistinguishable from noise. This implies that all three mechanisms may contribute to nonphotochemical quenching, with photoinhibition playing the major role as water content in leaves decreases.

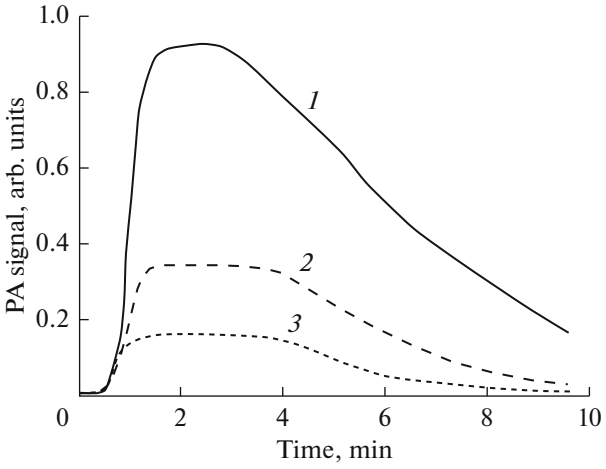

Fig. 5. Kinetics of changes in PA signal in leaves of indoor plant spathiphyllum at different water content levels: (*1*) 74; (*2*) 59; (*3*) 51%.

CONCLUSIONS

In this work, the physical characteristics of a PA spectrometer based on a three-colored RGB LED with emission wavelengths lying in different parts of the spectrum of photosynthesis-inducing radiation were investigated. By using the design that combines three emitters on a single base, which ensured identical profiles of the light beams, as well as the possibility of their independent modulation with different frequencies, the contribution of the photothermic and the photobaric PA signals, as well as their changes depending on the wavelength of light and the modulation frequency could be determined. The cross-sectional profiles of light absorption at different wavelengths and the corresponding photosynthetic activity in a leaf cross section, as well as the distributions of the generated photoacoustic waves were calculated. Additional illumination with saturating light was used to determine the photothermal and the photobaric components of the PA signal, which on the whole agreed with the values calculated using the thermophysical parameters of the medium concerned. Comparative analysis of the reflection spectra, the share of the photobaric component, and the quantum efficiency of the primary photochemical reaction in yellowing leaves in autumn indicated that there exists a time discrepancy in the degradation of different protein complexes involved in photosynthesis.

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