Reflectance Spectra of Leaves and Fruits during Their Development and Senescence and under Stress


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Abstract—The results of the authors' work on the application of reflectance spectroscopy for diagnostics of the physiological state of plants and plant injury are summarized. The problems of nondestructive measurement of chlorophyll content and following the course of autumnal leaf senescence, ripening of fruits, tissue browning, chlorophyll photohydration, and photodestructive events in situ are discussed.

Key words: Acer platanoides - Coleus blumei - Citrus limon - Malus domestica - Zea mays - senescence - stress - reflectance spectroscopy - chlorophylls - carotenoids - flavonoids

INTRODUCTION

The normal development of plants, their senescence, and the effects of unfavorable environmental conditions are accompanied by changes in the content and composition of plant pigments that determine leaf color. Visible manifestations of alterations in pigment content and optical properties of plant tissues, such as chlorosis, yellowing, necrosis, browning, etc., were generally used to characterize the state of a plant. Changes in leaf and fruit color are mainly due to transformations of major photosynthetic pigments: chlorophylls and carotenoids [1–8]. Plant color is also determined by the presence of other colored substances (flavonoids, anthocyanins, tannins, oxidation products of phenolic compounds, etc.) [1, 2, 9, 10].

Attempts to apply nondestructive methods of optical spectroscopy for the objective assessment of the physiological state of a plant have been undertaken for several decades. Considerable impetus was given to these attempts over the last two decades by the elaboration of remote sensing, including aerospace observations [11–13]. However, despite apparent advances in this sphere, it was believed that reflectance spectra do not carry sufficient information [14] as they detect only dramatic changes in plant condition. This can be related to the fact that leaves are adapted for efficient utilization of photosynthetically active radiation and have a complex anatomy, which is characterized by strong light scattering varying in particular intracellular structures, and a high pigment content. As a result, leaves manifest a low reflectance in the visible part of the spectrum (see [15–18] for details). Nevertheless, in recent years, a better understanding of the optical properties of leaves and in vivo pigment spectroscopy [18–21] created the necessary conditions for a more successful solution to these problems.

This work presents our data on changes in reflectance spectra during leaf senescence and fruit ripening and upon plant injury. We also describe some approaches that can be used for the assessment of plant state, detection of the type of injury, and quantitative interpretation of data.

MATERIALS AND METHODS

Leaves of deciduous trees (Norway maple, Acer platanoides L., and horse chestnut, Aesculus hippocastanum L.) were collected in a park near the Faculty of Biology of the Moscow State University during the entire growth season (from spring to late autumn) in the years 1992–1996. Maize (Zea mays L., cv. Odesskaya-10) and potato (Solanum tuberosum L., cv. Lorkh) plants were grown under field conditions and examined in late June–early July 1996. We also used leaves of the house plant Coleus blumei Benth. Leaf senescence was induced by placing leaves in darkness. Before the experiments, the leaves were washed with a diluted permanganate solution and then rinsed with water several times. The leaves were incubated at 25°C in sterilized petri dishes on moistened filter paper.
Lemon (*Citrus limon* Burm.) fruits, cvs. Pavlovskii and Novogruzinski, were collected from trees grown in a greenhouse under natural illumination at 20°C.

Pigment bleaching in the lemon fruits was induced by illuminating (0.25 W/cm²) them from a light source with a KGM 150/24 tungsten–halogen lamp through a 5-cm water filter and a 5-mm BS-S cut-off filter (see [22] for details).

Apple (*Malus domestica* Borkh., cv. Antonovka obyknovennaya) fruits were grown near Michurinsk (Tambov oblast), brought to Moscow within 1–2 days, and immediately analyzed. Postharvest ripening of apples was followed during their subsequent storage at 20°C.

To extract pigments, leaves were rapidly ground with a mortar and pestle in acetone or methanol, with calcium carbonate added to prevent phaeophytinization. Homogenates were centrifuged for 3–4 min in glass tubes at 3000 g. The resulting extracts were immediately assayed spectrophotometrically. Absorption coefficients of chlorophyll and carotenoids were taken from [23].

Reflectance and transmittance spectra were measured at a resolution of 2 nm with a double-beam 150–20 spectrophotometer (Hitachi, Japan) equipped with an integrating sphere and interfaced with a personal computer. Reflectance spectra were measured with barium sulfate as a standard. Leaves were placed on a black velvetenv background, which has a reflectance of less than 0.5% over the entire spectral range of measurements (see [19] for details). Reflectance coefficients ($R$) were represented as an $I_R/I_0$ ratio, where $I_R$ and $I_0$ are the intensities of the radiation reflected by the sample and BaSO₄, respectively. Kubelka–Munk remission functions [24] $f(R_m) = (1 - R_m)^2/2R_m$ were calculated assuming that $R_m$ corresponds to $R$.

**RESULTS AND DISCUSSION**

*General Characteristics of the Reflectance Spectra of Green and Senescing Leaves*

Figure 1 shows changes in the reflectance spectra of maple (Fig. 1a) and *Coleus* (Fig. 1b) leaves throughout their senescence, from maturity, when their chlorophyll content is maximal, to the late stages of senescence. The leaves exhibited the highest reflectance in the near IR region (above 750 nm), where chlorophyll absorption is low. At high chlorophyll concentrations, leaves of both plant species had a low reflectance (0.05–0.06) at the red maximum of chlorophyll $a$ absorption (near 678 nm) and in the absorption band at 350–480 nm (where both chlorophylls and carotenoids absorb). Reflectance in this region only slightly changed with a decrease in chlorophyll concentration down to 10–15 nmol/cm², which can be related to the saturation of light absorption by leaf pigments even at low concentrations [19]. Further destruction of chlorophylls in the course of leaf senescence was accompanied by increasing resolution of the reflectance spectra, in which absorption bands of chlorophylls $a$ (near 590 and 625 nm) and $b$ (near 655 nm) appeared. At these stages, the reflectance spectra of *Coleus* leaves exhibited bands at 440 nm (chlorophyll absorption) and 470–490 nm (chlorophyll $b$ and carotenoids). Leaves in which chlorophyll was almost completely destroyed had a high and constant reflectance up to 550 nm. An intense absorption by carotenoids (peak around 480–486 nm) was clearly seen in yellow leaves of maple (Fig. 1a).

Similar changes in optical spectra were previously reported for autumnal leaves of many deciduous trees that retain a considerable amount of carotenoids during autumnal senescence [19, 25–27]. Senescent leaves of *Coleus* were grayish white before abscission, and their reflectance spectra exhibited only weak carotenoid bands (Fig. 1b). The destruction of carotenoids in *Coleus* leaves correlated well with chlorophyll destruction ($r^2 = 0.96$).

It should also be noted that the reflectance of maple and *Coleus* leaves, with their low chlorophyll content, sharply decreased at wavelengths above 430 nm due to
the presence of compounds that absorb in the near ultraviolet region.

**Determination of Leaf Chlorophyll Content from Reflectance Spectra**

Previously, we investigated the possibility of determining chlorophyll content from reflectance spectra, using fully expanded young and senescent leaves of several deciduous trees and tobacco plants [19–21, 28–30]. We found that two zones in the reflectance spectra in the visible region—a broad band around 550–600 nm and a narrow band around 700 nm—were the most sensitive to changes in leaf chlorophyll content. We suggested several indices, which were based on leaf reflectance in the spectral regions that were sensitive and insensitive to changes in chlorophyll content. These indices were more sensitive and changed linearly in a wider range of leaf chlorophyll content (from dark-green to yellow-colored leaves) than previously suggested algorithms [19–21, 28–30]. Figure 2 shows the dependence of one of these indices \( R_{550} / R_{550} \) on chlorophyll content in maize leaves, a monocot C₄ plant with an anatomy markedly different from that of dicots [31]. As in the case of the previously examined plant species, this dependence was fairly linear, which allowed us to determine chlorophyll content in maize leaves with a standard error of 3.3 nmol/cm² over a wide range (up to 45 nmol/cm²). We demonstrated that the algorithms thus developed can be applied in the satellite monitoring systems [30, 32, 33].

In addition, the position of the peak at the slope of the reflectance spectrum in the red region (red edge) can be used for quantitative estimation of these pigments because it is closely correlated with the reflectance at 700 nm [20].

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**Leaf Senescence and Fruit Ripening**

Often (but not always), chlorophyll destruction in senescing leaves and ripening fruits is not accompanied by simultaneous carotenoid destruction; as a result, the characteristic yellow color of leaves is developed [1, 6–8]. This is related to the fact that, even at a low content of carotenoids, their contribution to absorbance increases due to the disappearance of green pigments and higher light penetration into a leaf [7]. As previously hypothesized [6, 7], the retention of carotenoids during plant senescence represents a mechanism for protecting cell structures from the photodynamic effects of blue light.

As noted above, the band at 550 nm in the reflectance spectrum \( R_{550} / R_{550} \) highly correlates with the chlorophyll content. Hence, carotenoid absorption should manifest itself at shorter wavelengths. This is also indicated by the reflectance spectra of yellow autumnal leaves (Fig. 1a), which exhibit clear-cut absorption bands of carotenoids, peaking at 460 and 486 nm (the minima in the reflectance spectra).

Previously [30], by comparing reflectance spectra of the green leaves of diverse plant species, we found a high correlation between reflectance coefficients at 678 nm (chlorophyll absorption) and 500 nm (carotenoid and chlorophyll absorption). This correlation can be explained by the saturation of pigment absorption in these spectral regions [19, 28, 30]. The linearity of this dependence was broken in senescing leaves ([30], Fig. 3). Figure 3a shows that the reflectance at 500 nm did not increase as chlorophyll disappeared \( R_{678} > 0.07–0.08 \) from the autumnal leaves of maple (see also Fig. 1a) and horse chestnut and remained at a low level throughout the entire period of disappearance of green pigments. In lemon fruits, which exhibit higher reflectance over the whole visible region (due to the high reflectance coefficient of albedo), the dependence of \( R_{500} \) on \( R_{678} \) remained linear to higher \( R_{678} \) levels (=0.25). However, further destruction of chlorophyll in the course of fruit ripening was accompanied by a marked decrease in \( R_{500} \) (Fig. 3a). Unlike maple and chestnut leaves, Coleus leaves were characterized by simultaneous degradation of chlorophylls and carotenoids. In these leaves, the linear dependence of \( R_{500} \) on \( R_{678} \) was retained at higher \( R_{678} \) values, and the reflectance coefficient at 500 nm increased until the complete destruction of chlorophyll.

The \( R_{500} / R_{678} \) ratio only slightly varied in diverse plants (between 1.02 and 1.3) at chlorophyll concentrations above 20 nmol/cm² (Fig. 3b). This agrees well with the high correlation between \( R_{500} \) and \( R_{678} \) at low values of the reflectance coefficient in green leaves (Fig. 3a). In Coleus leaves, this ratio was high even at low chlorophyll concentrations (2–3 nmol/cm²) and remained at or above 0.75 when virtually all chlorophyll disappeared. In the autumnal leaves of maple and horse chestnut, the \( R_{500} / R_{678} \) ratio drastically decreased at a chlorophyll content of about 15–20 nmol/cm². In yellow leaves, the \( R_{500} / R_{678} \) ratio was as low as 0.2–0.3.
Fig. 3. Changes in reflectance coefficients at 500 and 678 nm during the complete life cycle of (squares) maple, (circle) chestnut, and (triangle) Coleus leaves and (crossed squares) lemon (cv. Pavlovskii) fruits.

(a) The dependence of \( R_{500} \) on \( R_{678} \). (b) The dependence of the ratio \( R_{500}/R_{678} \) on leaf chlorophyll content.

These changes could indicate an imbalance in the catabolism of carotenoids and chlorophylls and, therefore, can be used as early indicators of leaf senescence.

Chlorophyll destruction and the increase in carotenoid content in apple peel, which occur during climacteric increase in the respiration rate, provide a reliable indicator of fruit ripeness [2, 3]. Pigment changes in ripening and postharvest ripening apple fruits were characterized by the changes in the 486-nm band, which reflects mainly carotenoid and chlorophyll \( b \) absorption. Figure 4a shows that, over a wide range of reflectance coefficient values (0.15 to 0.38), the increase in \( R_{500} \) in \textit{in situ} ripening fruits was matched by an approximately proportional increase in \( R_{486} \). Then, the increase in \( R_{486} \) somewhat slowed down. In fruits postharvest ripening at 20°C, \( R_{678} \) remained at the same level, whereas \( R_{500} \) attained lower values. Based on the published data [2, 3], these changes can be related to enhanced carotenoid synthesis in ripening apple fruits.

We used the \( R_{486}/R_{678} \) ratio to characterize the fruit postharvest ripening rate. As seen from Fig. 4b, this ratio declined during postharvest ripening. It should be noted that, in fruits collected before August 11, this decline was preceded by a 6- to 7-day lag period. This lag shortened in fruits collected at later dates (Fig. 4b, curve 2) and was absent in the case of fruits collected on August 31 and September 4 (curves 3 and 4). The \( R_{486}/R_{678} \) ratio was much lower and decreased faster in
Tissue Browning

The development of a characteristic brown color of plant cells and tissues is typical of mechanical damage, senescence, and hypersensitive response to pathogenic microorganisms and presents a characteristic manifestation of some infectious diseases and physiological disorders. The development of this coloration is believed to result from damage to plastids, tonoplast, and other membranous structures, causing decompartmentation and oxidation of some polyphenolic compounds by the vacuolar polyphenol oxidase, accompanied by the formation of polymeric melanin-like pigments [9, 10, 26, 36, 37].

Hence, we have examined browning of detached aging leaves of some plants. Very rapid (within 4–5 days) and pronounced browning was observed in potato and maize leaves, whereas, in Coleus leaves and the autumnal maple leaves, there were no signs of chlorophyll degradation and browning at least for 30 days (data not shown).

Browning of the autumnal maple leaves was observed already on the third day after placing the leaves in darkness. It was accompanied by a considerable progressive decrease in the reflectance coefficient in the visible spectral region (Fig. 5a). This was clearly manifested in a decrease in the reflectance coefficient in the near IR region. The products of polyphenol oxidation are characterized by unresolved absorption, which monotonically increased from the red to the blue region [37]. Their formation led to the loss from the reflectance spectrum between 500 and 750 nm of characteristic patterns that are typical of freshly collected leaves until the late stages of chlorophyll degradation (Fig. 1).

It is evident that the previously described methods for quantitative determination of chlorophyll content from optical spectra are inapplicable in this case or must be corrected.

Previously, we found [30] a correlation between the $R_{550}$ and $R_{700}$ (correlation coefficient $r = 0.98$, Fig. 5b, curve 1) in the reflectance spectra of healthy leaves of diverse plants, apparently due to the similar sensitivity of these indices to chlorophyll content [19, 28–30]. This apparently general property of reflectance spectra makes it possible to detect tissue browning. Thus, browning of senescing in darkness and autumnal leaves, differing in their chlorophyll content, was accompanied by a deviation from the linearity of the dependence of $R_{550}$ on $R_{700}$, which is characteristic of the intact leaves of this plant (Fig. 5b). Thus, this property can be used to estimate the extent of tissue browning and as a criterion of the absence of other pigments, which could interfere with chlorophyll determination from reflectance spectra.

Chlorophyll Pheophytinization

Chloroplasts of higher plants contain a certain amount of pheophytin $a$, which functions in the reaction centers. Chlorophyll pheophytinization (one of the

the fruits collected on the latter date than in the fruits collected on earlier dates.

A similar decrease in the reflectance coefficient at 500 nm, relative to that at 678 nm, was also observed during lemon fruit ripening (Fig. 3a). However, these changes were apparently related to both carotenoids and other yellow pigments of a flavonoid nature (anthoclores), which are present in high amounts in the vacuoles of lemon fruit cells [34, 35].

Thus, our data show that the band at 480–500 nm is sensitive to changes in the content of carotenoids and flavonoids. The ratio of reflectance coefficients in this region and at 678 nm can be used to estimate the rate of pigment transformation in senescing leaves and ripening fruits.
Fig. 6. Changes in the reflectance spectra of lemon (cv. Novogruzinskii) fruits frozen at -18°C for 14 h and thawed. C—control fruits; numbers indicate the time (h) of incubation after thawing. Heavy lines show the spectra of control fruits and 6-7 h after thawing. The inset represents an enlarged fragment of the figure.

The spectrum of the standard deviation of the reflectance of frozen and thawed lemon fruits (Fig. 7) drastically differed from that of green leaves [19] and was characterized by the presence of several narrow bands with high (at 542, 596, 628, and 696 nm) or low (at 516, 562, 612, and 668 nm) dispersion. These spectral ranges can be used for the in vivo estimation of the extent of chlorophyll pheophytinization in plants.

Fig. 7. Spectrum of standard deviation of lemon fruit reflectance after freezing and thawing (see Fig. 6).

The curve is plotted using fruit reflectance spectra measured every hour for 8 h after thawing.
bleaching [4, 5, 39]. This process belongs to the type II reactions of chlorophyll degradation [4, 5]. The probability of photooxidative reactions increases during plant senescence [6, 7].

Even intense illumination with visible light did not change the reflectance spectra of green lemon fruits, which can be explained by the high activity of antioxidative systems [39]. However, after a loss of chlorophyll in the course of natural senescence, pigments could be bleached by light of a moderate intensity (Fig. 8a). This bleaching was accompanied by a marked decrease in the $f(R_n)$ value in the red peak and between 460 and 550 nm. As seen from the spectra, there was no sign of chlorophyll phophorylation or tissue browning (spectral patterns in the green and near IR regions, respectively) during the pigment bleaching. The spectrum of a standard deviation of $f(R_n)$ was close to the absorbance spectrum of the isolated chloroplasts, indicating that chlorophylls and carotenoids were selectively degraded by light, despite a significant contribution of flavonoids to absorbance in the blue region.

Some spectral characteristics of fruit reflectance, which are typical of ripening and light-induced bleaching of the pigments in lemon fruits, are compared in Figs. 9a and 9b. Chlorophyll content (absissa) was characterized by the $R_{750}/R_{700}$ ratio, which was proportional to the content of this pigment [19–21]. With chlorophyll destruction, light induced a faster destruction of carotenoids (the increase in reflectance at 476 nm, Fig. 9a) as compared to that in ripening fruits. These differences were most pronounced when the reflectance in the 476- to 500-nm region drastically decreased in ripening fruits (cf. Figs. 3a and 4a). These observations agree with the published data [39] on the high sensitivity of carotenoids under conditions of photooxidative stress.

The light-induced degradation of pigments changed the chlorophyll absorbance spectra in the red spectral region. In the naturally senescing leaves [19] and the ripening lemon fruits (Fig. 9b), the dependence of minimum reflectance at 678 nm on chlorophyll content.

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**Photooxidative Degradation of Pigments**

Under oxidative stress, plant damage is often due to photodestruction, which is accompanied by pigment
(R₁₅₀/R₁₀₀) is described by a nearly hyperbolic function, which is due to the saturation of light absorption. In fruits differing in their initial chlorophyll content, these dependences were closer to linear under conditions of photodestruction (Fig. 9b, curves 2-4). We suppose that a faster pigment bleaching in the chloroplasts located near the leaf surface, as compared to those positioned in deeper layers and screened by chlorophyll, can explain these differences. As a result, the absorbance of light-exposed tissues at the chlorophyll peak is saturated at lower pigment concentrations. Thus, our data indicate that the changes in reflectance spectra that accompany pigment photodestruction can be distinguished from those during natural senescence.

In conclusion, it should be noted that, however complicated, the analysis of reflectance spectra can provide interesting information about the physiological state of plants and the course of natural and stress-induced senescence and allow for in situ diagnosis of different types of injuries.

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