Plant Stress Detection by Reflectance and Fluorescence

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OPTICAL PROPERTIES OF LEAVES AND PLANTS

Incident light will either be absorbed by the leaf, reflected from the leaf surface, or transmitted unabsorbed through the leaf. Reflectance and transmittance spectra of leaves can easily be measured using conventional spectrophotometers with an integrating sphere. From the latter the absorption spectra are calculated. All three types of optical spectra are largely dependent on the pigment content (chlorophylls, carotenoids) and to a minor extent also on the morphology and anatomy of leaves such as size, form, and arrangement of cells and aerial interspaces as well as on the roughness, smoothness, and cover (waxes, hairs) of the leaf surface, which influences the light scattering.

At a low content of photosynthetic pigments in the leaf, the absorption of incident light is low, and the reflectance and transmission are high. The absorption spectrum shows maxima in the blue spectral region (in vivo absorption bands of chlorophylls and carotenoids) and in the red spectral region around 670 to 680 nm absorption bands of chlorophylls, whereas the green-orange light is largely reflected and transmitted. In fully green leaves, however, the absorption of light is very high, whereas transmission and reflectance are low and predominantly restricted to the green spectral region. As a consequence, the leaves appear green to our eyes. The changes in reflectance spectra with increasing chlorophyll content are shown in Figure 1.

Absorption and transmittance spectra of leaves and plants can only be measured in direct contact with the spectrophotometer. In contrast, reflectance spectra can also be detected from a distance and are thus applied in remote sensing of the state of health of plants and in the assessment of the density of the green vegetation cover of the earth. This is done by measuring the reflectance of sunlight by airborne systems and satellites. Particular vegetation indices, based on the reflectance signals in the green, red, and near infrared, had been developed for this purpose and applied in the past. 1–5

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Financial support from the European Union, Brussels, within the INCO-COPERNICUS and the INTERREG II programs is gratefully acknowledged.

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Some papers given at the Stress of Life Congress dealt with the improvement and better evaluation of the reflectance signatures of plants with respect to the remote sensing of the vegetation cover and the detection of stress and strain conditions in plants. Analyzed were stress-induced changes in the composition of photosynthetic pigments, detection of onset of stress-induced senescence and partial chlorophyll breakdown (photooxidative processes), as well as stress-induced synthesis of red anthocyanin pigments, all of which change the optical reflectance signatures of leaves.6

A basic problem in remote sensing is the fact that the presently applied standard vegetation index NDVI5 is insufficiently sensitive to changes in medium and high pigment content1,2,4 (FIG. 2a). For this reason several groups have attempted to develop new vegetation indices that are more sensitive and can serve as indicators of the early stages of plant stress, senescence, and disease. For this purpose, high spectral resolution measurements of transmittance and reflectance spectra were performed in the range of 400 to 1100 nm with a parallel determination of the leaves’ pigment content.4,7,8 From these investigations, new vegetation indices were developed using reflectances that correspond to wavelengths with maximal and minimal sensitivity to variation in chlorophyll content. Maximal sensitivity of reflectance to chlorophyll content was found near 550 nm and 700 nm.4,8 Two new indices were established on the basis of these two reflectance signatures, which are directly proportional to the chlorophyll content. These are the Narrow-Band Vegetation Indices (NBVI): R750/R700 and R750/R550. The NBVI based on R750/R700 is shown in FIGURE 2c. To these was added a third new index, the “green” NDVI.9

In remote sensing, reflectance signatures in the visible and the near infrared are used to discriminate vegetation from other objects (TABLE 1). Most of these para-
meters reflect the absorption of chlorophylls and thus can be taken as indicators of the greenness or as indicators of long-term stress, which reduces the level of chlorophylls. When the reflectance \( r \) in the red absorption maximum of chlorophyll is related to the reflectance in the near infrared \( \text{nir} \) one does not expect and


![FIGURE 2. Differential sensitivity of several vegetation indices based on reflectance measurements to the chlorophyll content of a bean leaf \( \text{Phaseolus vulgaris L.} \). (a) Normalized Difference Vegetation Index (NDVI)\(^5\) (no change at medium or high chlorophyll content), (b) Chlorophyll Index \( \text{cc} \)^\(^2\) (good linear correlation), (c) Inflection Point (IP)\(^3,4\) (good linear correlation with chlorophyll content), (d) the new Narrow-Band Vegetation Index (NBVI)\(^4\) (good linear correlation). The calculation of these vegetation indices is given in TABLE 1.](image)

### TABLE 1. Parameters for the Classification of Vegetation from Reflectance Signatures in the Visible and the Near Infrared\(^a\)

<table>
<thead>
<tr>
<th>Vegetation Index (^{a})</th>
<th>Formula</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ratio Vegetation Index (^{a})</td>
<td>( \text{RVI} = \frac{r}{\text{nir}} )</td>
</tr>
<tr>
<td>Normalized Difference Vegetation Index (^{a})</td>
<td>( \text{NDVI} = \frac{(\text{nir} - r)}{(\text{nir} + r)} )</td>
</tr>
<tr>
<td>Inflection point of the red edge (^{3,4})</td>
<td>( \text{IP} = \text{inflection point of the rise to the NIR} ) (intersection of the second derivative of the reflectance spectrum with the ( x )-axis)</td>
</tr>
<tr>
<td>Chlorophyll Index (^2)</td>
<td>( \text{cc} = \log \left( \frac{R800}{R550} \right) )</td>
</tr>
<tr>
<td>Narrow-Band Vegetation Index (^4)</td>
<td>( \text{NBVI} = \frac{R750}{R700} )</td>
</tr>
<tr>
<td>“Green” Normalized Difference Vegetation Index (^*)</td>
<td>( \text{“Green” NDVI} = \frac{(\text{nir} - g)}{(\text{nir} + g)} )</td>
</tr>
</tbody>
</table>

\( ^{a}\) Present in chronological order. Abbreviations: \( r \), reflectance in the red absorption maximum around 680 nm; \( \text{nir} \), reflectance in the near infrared around 800 nm; \( g \), reflectance in the green absorption minimum around 550 nm; \( R800, R750, R700, R550 \), reflectance at the wavelengths 800, 750, 700, and 550 nm.
find a linear correlation with chlorophyll content (1) because in a leaf tissue the red reflectance minimum is saturated already at low chlorophyll content, (2) because the reflectance at the leaf surface can increase the reflectance in all parts of the visible spectrum, and (3) because if the leaf were transparent like a solution (which it is not) the chlorophyll content would be proportional only to the absorbance (= \( \log I_0 / I \), \( I_0 \) = light intensity incident on the sample, \( I \) = light intensity transmitted through the sample)—according to the Lambert-Beer law—and not to the percentage of absorption related to the percentage of reflectance. Thus the hitherto applied NDVI clearly saturates very early with increasing chlorophyll content of the leaf (Fig. 2a) and is not suitable for remote sensing at a high green vegetation cover.

Also, the chlorophyll-index \( c_c = \log (R_{800} / R_{550}) \) shows a good linear correlation to the chlorophyll content of a leaf (Fig. 2b). The reflectance at 800 nm can be taken as a basis reference of no chlorophyll absorption, and the reflectance at 550 nm is in the range of low chlorophyll absorption and is better correlated to the chlorophyll content because it does not saturate so early as the value in the absorption maximum of chlorophyll.

The inflection point (IP) at the red edge of the reflectance spectrum shifts towards longer wavelengths with increasing chlorophyll content due to the broadening of the absorption band in the red caused by the detour effect and the sieve effect of light penetrating the leaf. The IP is well linearly correlated with the chlorophyll content of the leaf (Fig. 2c). Using the NBVI at \( R_{750}/R_{700} \) (Fig. 2d), being based on the reflectance measurements near 700 nm, one avoids the calculation of the second derivative of the reflectance spectrum (for the determination of the wavelength position of the IP) and obtains direct information on the chlorophyll content.

**CHLOROPHYLL FLUORESCENCE KINETICS AND PARAMETERS**

Time-resolved chlorophyll fluorescence measurements and saturation pulse quenching analyses (yielding particular chlorophyll fluorescence parameters, coefficients and ratios) have become sophisticated techniques to detect plant stress. The application of such techniques allowed to detect high-light stress and photo-inhibition, to define acclimation and stress tolerance, the sensitivity of wheat genotypes and pea to drought and heat, the effect of cadmium and lead as well as paraquat resistance and virus infections.

More recently the polyphasic chlorophyll fluorescence rise curve has been resolved by R. Strasser in further detail. This O-J-I-P phase of the fluorescence rise changes considerably as a result of various kinds of stress (Fig. 3). These phases are particularly seen at a logarithmic time scale and are not readily detectable in the linear plot of the fluorescence rise. The rise from O-J represents the reduction of the first semiquinone acceptor \( Q_a \) to its reduced form \( Q_a^- \). The level I is suggested to be related to a heterogeneity of processes in the filling up of the plastoquinone pool (e.g., the reduction of the second quinone acceptor \( Q_b \) to \( Q_b^- \)). P is reached when all the plastoquinone molecules are reduced to plastoquinone (PQ → PQH). If the photosynthetic electron transport is inhibited by the herbicide diuron, only the rise via O to J occurs but is then at a higher level.

Although such fluorescence measurements can provide valuable information on the stress and strain exposure of plants, there is the disadvantage that they represent point data measurements and can only be applied with certainty when the stress-induced changes or damage have already been established. Very early
stress indicators are, however, local disturbances in chlorophyll fluorescence emission, for example, at the leaf rim or at isolated leaf points, but these are overlooked when using point data measurements. This problem can, however, be overcome by the laser-induced fluorescence (LIF) imaging technique, which simultaneously measures the LIF signatures of all leaf points of the whole leaf area or plant canopy.20–22

FIGURE 3. Chlorophyll a fluorescence rise obtained after illumination of a dark-adapted leaf, here plotted on a linear (upper part) and on a logarithmic (lower part) time scale, showing the phases O (ground fluorescence), J, I, and P (maximum fluorescence).20,21
The fluorescence emission spectrum (λ_{exc} = 355 nm) of a green leaf exhibits a maximum in the blue region near 440 to 450 nm (termed F440 or F450), a shoulder in the green region between 520 to 530 nm (termed F520 or F530) as well as the red and far-red chlorophyll fluorescences near 690 nm (F690) or in the range of 735 to 740 nm (termed F735 or F740) as shown in Figure 4. In green leaves the blue-green fluorescence is primarily emitted by cinnamic acids of the cell walls of the chlorophyll-free epidermis cells and of leaf veins to which further secondary plant phenolics and flavonols in the vacuoles can contribute. The red and far-red fluorescences, in turn, are emitted by chlorophyll a in the chloroplasts of the leaves’ mesophyll cells.

In contrast to dicotyledonous plants (e.g. tobacco), where the blue-green fluorescence is often lower than the red and far-red chlorophyll fluorescence, the blue-green fluorescence yield of the grasses (Poaceae) is much higher (Fig. 5). Correspondingly, the fluorescence ratios blue/red and blue/far-red of maize, wheat, and other Poaceae are much higher than that of dicot plants.

When field plants exposed to full sunlight are excited by UV-A radiation of 355 nm, the blue-green fluorescence is easily detectable, the red and far-red chlorophyll fluorescence yield is, however, very low and almost not detectable (Fig. 6, dotted line). This is due to the fact that the epidermis layer of field plants contains many UV-absorbing substances with the result that only a small percentage of the applied UV can penetrate through the epidermis into the green mesophyll cells to induce chlorophyll fluorescence. When the chlorophyll fluorescence is, however, excited by blue (430 nm) or red light (633 nm) a very high fluorescence yield is obtained, which shows the typical red and far-red fluorescence maxima of chlorophyll (Fig. 6).
In plants with mineral deficiencies, in particular nitrogen and magnesium deficiency, which cause lower chlorophyll and carotenoid content, the relative amounts of blue-green fluorescence and red + far-red chlorophyll fluorescence change. This results, for example, in maize at a progressed deficiency, in much lower values of the fluorescence ratios blue/red, blue/far-red, green/red, and green/far-red (TABLE 2). The fluorescence ratio blue/green is then also decreased. In contrast, the values of the chlorophyll fluorescence ratio red/far-red is significantly increased (TABLE 2). The fluorescence emission of the upper leaf sides of the bifacial leaves of dicot plants show a lower yield of blue, green, and red fluorescence than that of the lower leaf sides. This is also seen in the C₄ plant maize, which exhibits a "Kranz"-type anatomy (FIG. 6). This is caused by a higher partial reabsorption of the emitted blue, green, and red fluorescences by the higher levels of chlorophylls and carotenoids in the upper than in the lower leaf side.

**LASER-INDUCED FLUORESCENCE IMAGING**

Plants are exposed during their lifetimes to various kinds of natural and anthropogenic, biotic and abiotic stress constraints. In recent years it has been shown that the chlorophyll fluorescence ratio red/far-red (F690/F740) represents an excellent nondestructive indicator of stress-induced decreases in chlorophyll content. Further fluorescence ratios, such as plants' blue/red (F440/F690) and blue/far-red (F440/F740), in turn, are even more sensitive to changes in the environment and increase or decrease as a response to stress constraints. Both ratios, as determined via fluorescence emission spectra or via the new fluorescence imaging technique, can provide valuable information for stress detection and management.

**FIGURE 5.** Differential yield of blue-green fluorescence of a maize (*Poaceae*) and a tobacco (dicot plant) leaf with 355 nm excitation. These differences in blue-green fluorescence are also seen between other members of the grass family (wheat, barley, rice) and dicot plants; they reflect the much higher level of cinnamic acids in the cell walls of the *Poaceae* than in dicot plants.
imaging technique, thus allow a very early stress detection as is demonstrated here. Their monitoring by fluorescence imaging of whole leaves or plants permits very early stress detection in plants at a stage in which countermeasures can still be taken to overcome the stress-induced changes in order to avoid severe damage to the plants. Furthermore, a stress-induced decrease in chlorophyll content is detected by significant increases of the Chl fluorescence ratio red/far-red.

Although all fluorescence ratios can be determined from fluorescence emission spectra of leaves (point data measurements), the laser-induced fluorescence imaging is much superior because it simultaneously senses the fluorescence emission of the complete leaf (i.e., several hundred leaf points) or even of whole plants. This allows us to detect gradients and local disturbances in fluorescence yield and ratios that are very early indicators of stress events.

The Karlsruhe/Strasbourg fluorescence-imaging system consists of a Nd-YAK laser (excitation 355 nm). The laser beam is widened to excite the whole leaf area or plant. The fluorescence of all leaf parts, that is, several hundred leaf points, is simultaneously sensed in the blue, green, red, and far-red region by a CCD-camera (with gated image intensifier) as shown in Figure 7. The blue, green, red, and far-red fluorescence images as well as the fluorescence ratio images (such as blue/red, blue/far-red, or red/far-red) provide valuable and very early stress information on plants. The Karlsruhe/Strasbourg fluorescence imaging system works already in the near distance of 0.5 to 10 m and is presently complemented to a mobile system for sensing of the state of health of crop plants in the field. This fluorescence imaging technique can be further developed into a LIDAR fluorosensor for the physiological control of agricultural crops and forests from airborne systems.

The differences and changes of the fluorescence intensities in the blue, green, red, and far red spectral regions can be quantified by forming the fluorescence ratios.

### Table 2. Differences in the Fluorescence Ratios and Photosynthetic Pigments of a Green Leaf from Control Maize and a Yellowish-Green Leaf from Maize Grown with an Advanced Mineral Deficiency (Combined N-, K-, and Mg Deficiency)

<table>
<thead>
<tr>
<th>Fluorescence/Pigment</th>
<th>Green Leaf</th>
<th>Yellowish-Green Leaf</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Fluorescence ratios</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>blue/red F440/F690</td>
<td>21.4</td>
<td>3.1</td>
</tr>
<tr>
<td>blue/far-red F440/F740</td>
<td>27.5</td>
<td>6.0</td>
</tr>
<tr>
<td>green/red F530/F690</td>
<td>6.0</td>
<td>1.2</td>
</tr>
<tr>
<td>green/far-red F530/F740</td>
<td>7.7</td>
<td>2.3</td>
</tr>
<tr>
<td>blue/green F440/F530</td>
<td>3.6</td>
<td>2.6</td>
</tr>
<tr>
<td>red/far-red F690/F740</td>
<td>1.3</td>
<td>2.0</td>
</tr>
<tr>
<td><strong>Pigment content</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>chlorophylls a+b</td>
<td>33.2</td>
<td>7.5</td>
</tr>
<tr>
<td>carotenoids x+c</td>
<td>7.6</td>
<td>3.0</td>
</tr>
<tr>
<td><strong>Pigment ratios</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chl a/b</td>
<td>4.2</td>
<td>3.1</td>
</tr>
<tr>
<td>(a+b)/(x+c)</td>
<td>4.4</td>
<td>2.5</td>
</tr>
</tbody>
</table>

*a* The individual fluorescence intensities in the blue (F440), green (F530), red (F690), and far-red region (F740) were taken from fluorescence emission spectra (λ excitation 355 nm) measured with a Perkin-Elmer fluorometer. The total content of chlorophylls a+b and of carotenoids x-c (xanthophylls + carotenes) is given in µg per cm² leaf area. Mean of three determinations (standard deviation: < 5 % ratios and < 7 % pigment levels). The differences between the two maize plants were highly significant: *p* < 0.01.
This can be done from fluorescence emission spectra but in a better way from fluorescence ratio images, whereby the fluorescence information of several hundred leaf pixels is used for the mean values. This provides at an early stage of stress events highly significant values, even when the differences in fluorescence ratios are not very large. Some examples are given here for early nitrogen deficiency in maize, for herbicide inhibition of the photosynthetic apparatus, and for the attack of leaves by mites (FIGS. 8, 9, and 10).

**FIGURE 6.** Fluorescence emission spectra of the upper and lower leaf side of a fully grown maize leaf (chlorophyll content 33.2 µg cm⁻²). The chlorophyll fluorescence yield is much higher at an excitation at 430 nm and 633 nm than by UV radiation (355 nm). The blue-green fluorescence as well as the red + far-red chlorophyll fluorescence emissions are significantly higher in the lower than upper leaf side of maize. The absolute values of the chlorophyll fluorescence ratio F690/F740 depend on the excitation wavelength and are 1.29, 1.76, and 1.04 for the upper leaf side and 1.36, 1.76, and 1.25 for the lower leaf side when applying 355, 430, and 633 nm as excitation light.
FIGURE 7. Scheme of the Karlsruhe/Strasbourg laser-induced fluorescence imaging system LIFIS for near distance and remote sensing of leaves or plants. By applying appropriate filters, the fluorescence images are screened in the blue, green, red, and far-red spectral region. Because of its high resolution, the LIFIS allows us to detect even small local disturbances and stress-induced inhomogeneities in fluorescence emission as well as fluorescence gradients over the whole leaf area. This permits a very early stress diagnosis long before visual symptoms are detectable.

FIGURE 8. Increase in the fluorescence ratios blue/red (F440/F690) and blue/far-red (F440/F735) in a leaf of maize plant with beginning N deficiency compared to the control. Mean of three determinations. Significance: **p < 0.01.
Another new development, which represents a real breakthrough in our understanding of the emission of chlorophyll fluorescence, is the deconvolution of the red and far-red fluorescence emission spectrum of plants. This chlorophyll fluorescence reabsorption process considerably decreases the F690 band. In contrast, the far-red chlorophyll fluorescence emission band near 730 to 740 nm (termed F740 or F735) is little affected by this reabsorption process. As a consequence, the fluorescence ratio F690/F740 decreases with increasing chlorophyll content of leaves and is an excellent nondestructive indicator of the in vivo chlorophyll content of leaves.

The chlorophyll fluorescence emission should steadily increase with increasing chlorophyll content. This is, however, not the case because of the reabsorption of the 690 nm fluorescence as mentioned above. This problem has now been solved in a very easy way. By measuring the reflectance and transmittance spectra, the absorption spectra of leaves can be determined. From the latter one can calculate for each wavelength the relative amount of the actually emitted chlorophyll fluorescence that had been reabsorbed by the leaf’s chlorophyll. In this way the “actual” or “true” chlorophyll fluorescence emission could be retrieved.

**FIGURE 9.** Decline of the fluorescence ratios blue/red (F440/F690) and blue/far-red (F440/F740) in a green tobacco leaf treated with the herbicide diuron (10⁻⁵ M) via the lower leaf side. The Chl fluorescence ratio red/far-red (F690/F740) increased by 25% compared to the control. Fluorescence imaging (n = 5) with 300 pixels each. Significance: ***p < 0.001 and *p < 0.05.

**RETRIEVED “TRUE” CHLOROPHYLL FLUORESCENCE**
FIGURE 10. Increase in the fluorescence ratios blue/red \( (F_{440}/F_{690}) \) and blue/far-red \( (F_{440}/F_{740}) \) in bean leaves attacked by mites as compared to controls. Determined via fluorescence imaging \( (n = 10) \) with 200 pixels each. Significance: *** \( p < 0.001 \) and * \( p < 0.05 \).

FIGURE 11. Absorption spectra and measured chlorophyll fluorescence of a mature green beech leaf. "True" chlorophyll fluorescence was retrieved from the measured one, taking into account reabsorption of the emitted chlorophyll fluorescence by the leaf. The shape of the "true" fluorescence signal is very similar to the one recorded for the fluorescence emission spectrum of chlorophyll \( a \) in solution. The same spectral behavior of "true" fluorescence was observed for beech, wild wine, platanous and elm leaves in a wide range of pigment content from 7 to 65 \( \mu \text{m cm}^{-2} \).
The originally emitted “true” chlorophyll fluorescence (f) possesses a high emission maximum near 685 nm (f685), which in green leaves is about 10 times higher than the measurable F690 chlorophyll fluorescence.35 Furthermore, the emission maximum of the measured red chlorophyll fluorescence in the 690 nm range, which shifts with increasing chlorophyll content from 685 nm via 690 nm to 695 nm in dark green leaves, is maintained in the case of the retrieved true chlorophyll fluorescence at or near 685 nm. In this retrieved chlorophyll fluorescence, the 685 nm maximum is about 10 times higher than the far-red fluorescence maximum f740 (Fig. 11). In fact, in the true chlorophyll fluorescence emission spectrum, the f740, is only expressed as a low shoulder. Thus, the retrieved chlorophyll fluorescence emission spectra of leaves resembles very much the fluorescence emission spectra of diluted solutions of pure chlorophyll a.32,33,35 These results also demonstrate that the shape of the measurable chlorophyll fluorescence emission spectra is determined to about 95% by the chlorophyll content of leaves and that the leaf anatomy, morphology, and the differential cell arrangement, such as in sun and shade leaves of the beech, have only a very minor effect on the shape of the chlorophyll fluorescence emission spectrum of leaves. This is further documented by the fact that in the case of the retrieved fluorescence (f) the fluorescence ratio f685/f740 exhibits very high values in the range of 6 to 9 and is virtually independent of the chlorophyll content of the leaves.

SUMMARY

During their life cycle, plants are exposed to various kinds of stress constraints that are caused by natural and anthropogenic as well as biotic or abiotic stressors. An early stress diagnosis is an option in order to take immediate countermeasures to protect the crop plants and forest trees against damage. In recent years noninvasive, optical methods have gained much attention in stress detection in plants. These are passive reflectance measurements (sunlight reflectance) and active laser-induced fluorescence measurements. The progress made in both fields is summarized in this report, which also provides some basic information.

The main emphasis in the field of reflectance signatures was put on improved vegetation indices. In the field of fluorescence signals, the emphasis was on the screening of red and far-red chlorophyll fluorescence signatures and the sensing of the plants’ blue-green fluorescence. A technological innovation was the presentation of the laser-induced fluorescence (LIF) imaging technique, which includes blue-green fluorescence and sets a new standard in an early stress detection in plants. The fluorescence ratios blue/red (F440/F690) and blue/far-red (F440/F735) proved to be very sensitive indicators of ongoing stress events. This has been demonstrated by fluorescence emission spectra and the novel fluorescence imaging technique. High-light, water, and temperature stress as well as nitrogen deficiency, herbicide application, and attacks by mites and other predators can easily be monitored via increasing or decreasing fluorescence ratios and ratio images. In addition, for the first time a method of evaluation of the true chlorophyll fluorescence from joint reflectance and fluorescence measurements has been established that opens further possibilities for stress detection. Several of the presented optical reflectance and fluorescence techniques are also applicable for remote sensing of the state of health of terrestrial vegetation.
ACKNOWLEDGMENTS

We wish to thank Dr. N. Subhash for assistance in fluorescence measurements in maize, and Mrs. Doris Möller and Mrs. Inge Jansche for their excellent assistance during preparation of the manuscript.

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