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Analysis of Chlorophyll Fluorescence Spectra in Some Tropical Plants

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Laser-induced chlorophyll fluorescence (LICF) emission spectra of leaves of some tropical plants were measured using a compact fiber-optic fluorosensor with a continuous-wave blue diode laser as exciting source and an integrated digital spectrometer. Different chlorophyll-fluorescence signatures of light-green, fully-green, and yellow leaves were monitored at room temperature. Deconvolution procedure was used to determine fluorescence band position and width. Calibration of the fluorescence ratio F690/F730 relative to the 404 nm excitation is done from the curve-fitted parameter.

KEY WORDS: Chlorophyll-fluorescence emission spectra; chlorophyll-fluorescence ratio; chlorophyll content.

INTRODUCTION

In recent years there is a growing interest in laser remote-sensing techniques to assess the status of vegetation in environment [1]. Monitoring of vegetation by spectroscopic detection of electromagnetic radiation is a powerful noncontact and nondestructive method in the study of environment. In particular, laser induced fluorescence spectroscopy of terrestrial vegetation is an important aspect of active remote sensing which provides a specific tool for assessing vegetation damage and forest decline [1–3].

Within plant tissue, the light energy (in the region of 400–800 nm) absorbed by photosynthetic pigments (chlorophylls a, b, and carotenoids) is mainly used to drive the photosynthetic processes which provide chemical energy for plant's growth when under optimum conditions the largest part of the absorbed light energy is used for CO_2 fixation in the Calvin cycle [6]. Photosynthesis takes place in the chloroplasts in which the photosynthetic pigments are localized. Chlorophyll molecules (several hundred) are arranged into two groups of pigments known as photosystem I (PSI) and photosystem II (PSII). Each photosystem has 'antennae' chlorophyll molecules and a central chlorophyll molecule P700 and P680, respectively, where the numerals indicate the maximum of the absorption peak of the two species of chlorophyll *a* [4]. When an antennae chlorophyll molecule absorbs photons it transfers this energy to another nearby one until reaching the reaction center chlorophyll molecule.

Part of absorbed energy is lost during the migration from the pigment antenna to the reaction centers and can be dissipated by a variety of non-photochemical processes. Such processes include the emission of heat and re-emission of small but diagnostically significant amounts (2–5% in vivo) of the absorbed radiation. This re-emission which occurs at longer wavelength in the red and far-red regions is termed as Chlorophyll Fluorescence [4,7]. At room temperatures approximately 95% of the chlorophyll fluorescence signal observed is derived from chlorophyll molecules associated with (PSII) [8] and chlorophyll *a* is the only significant source of red Fluorescence in leaves [9]. This results of the highly efficient energy transfer from chlorophyll b to chlorophyll *a* due to the overlap of the chlorophyll *b* fluorescence peak and the chlorophyll *a* absorption peak in addition to the organized structure of the pigments.

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The indicator function of chlorophyll fluorescence arises from the fact that fluorescence emission is complementary to alternative pathways of de-excitation which are primarily photochemistry and heat dissipation. If photosynthetic processes are very active then red fluorescence at 690 nm as well as the fluorescence ratio (FR) of the chlorophyll red and far-red bands, noted F690/F735, will be low. Conversely, if these processes are inactive or impaired then red fluorescence and (FR) increase [4,13].

The capacity of a plant for photochemistry is limited and will depend on a range of factors including stresses caused by environmental conditions [10–12]. This fact enables chlorophyll fluorescence to be used as a standard method for investigating plant class differentiation, chlorophyll contents monitoring and plant stresses detection (water deficit, temperature, nutrient deficiency, polluting agents, attack by pathogens, etc.) [11–15]. Such studies require *a priori* a basic knowledge of fluorescence signature of leaves.

Here the laser induced chlorophyll fluorescence (LICF) of leaves of some tropical plants is measured at ambient temperature using a compact fiber-optic fluorosensor with a continuous-wave blue diode laser as exciting source and an integrated digital spectrometer. Measured fluorescence spectra were fitted with a linear combination of Gaussian spectral functions. For each Gaussian band, the fitting procedure separate out the red and far-red fluorescence spectra and provided the peak wavelength, the band width, the peak amplitude and the band area. The F690/F735 Fluorescence Intensity Ratio obtained from curve-fitted parameters is qualitatively correlated to the chlorophyll content in different plants.

MATERIALS AND METHODS

Trees and Plants Selected

Our experiment concerned leaves of four plant species (*Moringa oleifera*, *Azadirachta indica*, *Hibiscus subdariffa*, and *Adansonia digitata*) collected in August, at the beginning of the raining season, in the Botanical garden of Cheikh Anta DIOP University in Dakar-Senegal. Our interest in these plants is due to their local use as human nutrients (*Moringa oleifera* and *Adansonia digitata*, *Hibiscus subdarifa*), and also to their use as medicinal plants in research against malaria (*Azadirachta indica*) and in the treatment of Diabetes (*Moringa oleifera*).

LICF Measurements

The chlorophyll fluorescence was measured under steady-state conditions, i.e., 5 mn after onset of the excitation light. Excitation and sensing of the chlorophyll fluorescence were performed on each leaf surface, either upper or lower leaf side.

The experimental apparatus (showed in Fig. 1) is constituted by a compact fluorosensor [16] on the basis of a blue diode laser and an integrated spectrometer assembled at the Lund Institute of Technology by two of the authors during a workshop that held here [17].

The light source of the fluorosensor is a continuous-wave blue semiconductor laser from Nichia Corporation (type NLHV500) with nominal wavelength at 25°C of 404 nm and output power of 5 mW. The diode laser is driven by a power-supply (wavelength Electronics LDD 200-3M) with 9 V battery as input for decoupling from possible net transients. The diode laser is placed in a tube with a collimating lens (Geltech C230TM-A). The output light is cleaned up for broadband spontaneous emission using a narrow-band interference filter (CVI F25-400-4-0.5). About 99.5% of the energy is reflected by an appropriate dichroic beamsplitter (CVI) and transmitted through a fiber port lens assembly (Optic for research PAF-SMA-6-NUV-Z). The blue radiation is focused by the fiber port lens assembly into a 2 m long, 600 μm diameter fused silica optical fiber.

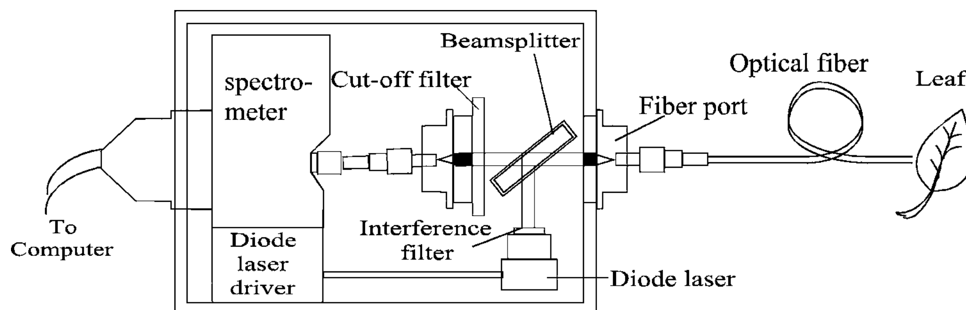

Fig. 1. Experimental arrangement for measurement of LICF spectra.

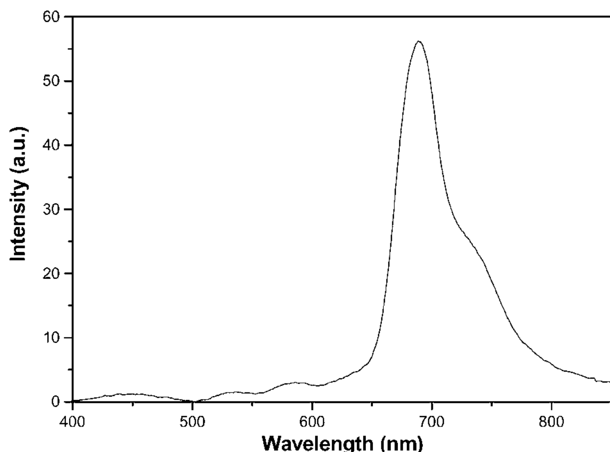

Fig. 2. Chlorophyll fluorescence emission spectrum of light-green leaf of *Moringa oleifera* excited at 404 nm and sensed from the upper leaf side 5 mn after unset of the light.

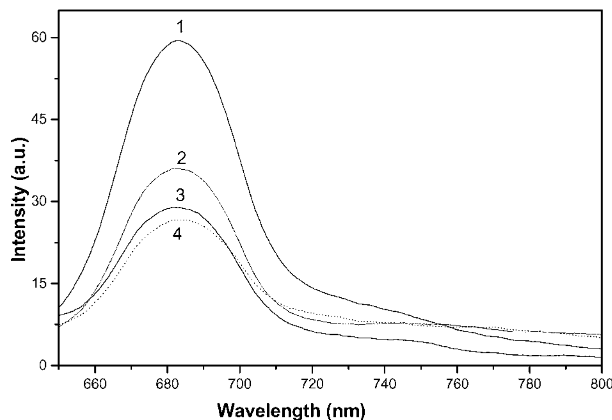

Fig. 4. Lower leaf side blue light excited chlorophyll fluorescence of light-green leaf of *Moringa oleifera* (1), *Adansonia digitata* (2), *Azadiracha indica* (3), and *Hibiscus sabdariffa* (4) yellow leaves; excited at 404 nm and sensed side in the steady state conditions.

Proper transmission was ensured by adjusting all optical elements in stable and adequate positions and aligning them to obtain a Gaussian intensity profile (tested by eye) at the end of the fiber, where about 1.2 mW of laser power is available.

Since the laser source is not pulsed and the detection is not gated, there is the risk that ambient light would add an undesired spectrum on top of the fluorescence spectrum. However, since the fiber is put in contact with the leaf surface the ambient light is effectively shadowed away by the fiber itself in most cases.

The Fluorescence induced in the leaf surface (either upper or lower leaf side) is conducted back through the same optical fiber. Since the spectrometer is fiber-coupled, the LICF light is focused by a fiber port into a short fiber which is connected to the 100 μm entrance slit of the miniature spectrometer (Ocean Optics S2000). The fixed grating (600 lines/mm—Blaze Wavelength 400 nm) disperses the light and a spectral region of about 350–1000 nm is captured on the 2048-element linear silicon CCD-array detector with a spectral resolution of about 4 nm. To avoid elastically backscattered diode laser light a Schott GG420 colored glass cut-off filter is placed behind the dichroic beamsplitter. The Miniature Fiber Optic Spectrometer communicates with a portable PC via an external analog-to-digital converter and is supported by OOIbase32TM, the Windows-based operating software.

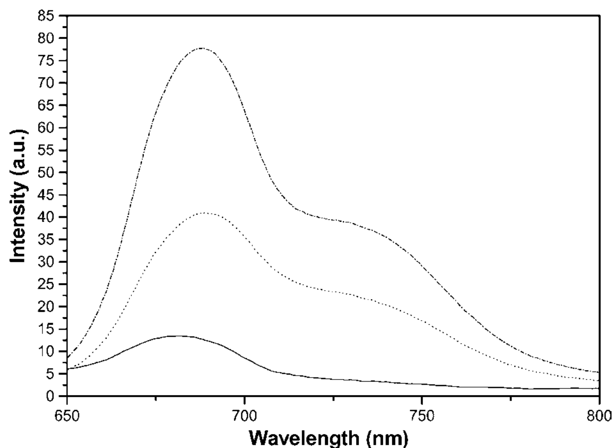

Fig. 3. Chlorophyll fluorescence emission spectra of yellow (1), light-green (2) and fully-green (3) leaves of *Azadiracha indica* excited at 404 nm and sensed from the upper leaf side in the steady state conditions.

All spectra presented in this paper were handled with appropriate software after transferred from the spectrometer.

RESULTS AND DISCUSSIONS

Fluorescence spectra of leaves with different chlorophyll content, light-green (L-G), fully-green (F-G), and yellow-green (Y-G) of four plants species, *Moringa oleifera*, *Adansonia digitata*, *Azadirachta indica*, and *Hibiscus subdariffa*, were recorded in August 2002, at the beginning of the raining season in Dakar, Senegal.

Chlorophyll fluorescence measurements, usually carried out with low irradiance light, taken at the upper leaf-side alone are not representative for the physiological situation of the whole leaf. Chlorophyll fluorescence signatures have to be determined as well from the lower

Table I. Upper Leaf Side Curve-Fit Parameters of *Adansonia digitata*, *Hibiscus sabdariffa*, *Moringa oleifera,* and *Azadiracha indica*

Plant	Band	λ_{peak} (nm)			Area (cm^2)			FWHM (cm^{-1})			Intensity		
		L-G	F-G	Y-G	L-G	F-G	Y-G	L-G	F-G	Y-G	L-G	D-G	Y-G
Adansonia digitata	Red	687	685	681	1190	1525	400	30.7	28.0	27.7	32.0	43.5	11.5
	Near- IR	728	727	758	3371	3447	1540	100.6	78.2	301.9	30.2	35.2	4.1
Hibiscus sabdariffa	Red	685	681	683	2651	4707	435	29.8	29.6	30.1	70.9	127.0	11.5
	Near- IR	721	719	727	2672	5072	1088	70.6	62.5	180.6	30.2	64.8	4.8
Moringa oleifera	Red	686	686	680	1443	748	416	29.4	28.0	30.3	39.2	21.3	10.9
	Near- IR	721	723	746	2521	2456	1622	80.3	79.7	239.3	25.1	24.6	5.4
Azadiracha indica	Red	685	685	683	976	2675	1471	29.1	30.9	180.4	26.8	69.0	6.5
	Near- IR	721	731	—	2047	1905	273	71.8	47.8	25.9	22.7	31.8	8.4

leaf side to correctly judge the physiological state of the leaf.

The shape of the fluorescence emission spectrum of leaves depends also on the wavelength of the excitation light [15]. Incident blue light is absorbed by carotenoids and by the chlorophylls of the chloroplast already at the upper part of the leaf mesophyll. The major part of the blue excited chlorophyll has to cover a short distance before it finally leaves the leaf at the epidermis and the chlorophyll fluorescence is only slightly reabsorbed by *in situ* chlorophyll. However, in the case of red light, which is only absorbed by chlorophylls but not by carotenoids, a substantial part of the excitation light penetrates deeper into the leaf mesophyll. This will generate more reabsorption of the red light induced chlorophyll fluorescence F690 and the shoulder F735 appear as a separate maximum with similar intensity [26].

When excited with blue radiation, plants exhibit a fluorescence emission spectrum in the blue-green region (400–550 nm), and in the red to far-red region (650–800 nm) as shown in Fig. 2. However, the blue-green fluorescence emission intensities when excited at 404 nm are too low to be used as fluorescence signatures [25]. The red fluorescence is characterized by a maximum in the red region (680–700 nm) and referred to as F690, and a shoulder in the far-red region (730–740) termed F735.

For very-low concentration of chlorophyll pigments as in light-green leaves, the fluorescence intensity will be roughly proportional to the amount of chlorophyll in the leaves. Fluorescence emission spectra of *Azadirachta Indica* light-green leaves (Fig. 3) shows one maximum in the red band centered at about 685 nm and a shoulder at about 720 nm. The expansion of the shoulder proceeds with increasing chlorophyll content. However, for higher concentrations, corresponding to fully-green to dark-green leaves the chlorophyll fluorescence will decrease considerably as it is confirmed here (Fig. 3). This can be explained by the partial overlapping of the absorption spectrum of green leaves with the fluorescence spectrum between 640 nm to about 710 nm [5,13], which will induce a substantial proportion of the fluorescence reabsorption by pigments in the leaves. The degree of fluorescence decrease is higher in the 690 nm region, due to the predominant reabsorption of the shorter wavelength fluorescence by chlorophylls.

In Yellow leaves (Fig. 4) the spectrum exhibits a low maximum near 682 nm and a strong reduction in the F740 nm fluorescence shoulder in relation

Table II. Lower Leaf Side Curve-Fit Parameters of *Adansonia digitata*, *Hibiscus sabdariffa*, *Moringa oleifera,* and *Azadiracha indica*

Plant	Band	λ_{peak} (nm)			Area (cm^2)			FWHM (cm^{-1})			Intensity		
		L-G	F-G	Y-G	L-G	F-G	Y-G	L-G	F-G	Y-G	L-G	D-G	Y-G
Adansonia digitata	Red	685	685	683	2318	2379	1067	29.7	29.2	28.1	62.3	64.9	30.3
	Near- IR	721	722	725	3376	4258	1826	71.9	72.3	193.2	37.5	47.0	7.5
Hibiscus sabdariffa	Red	685	685	684	1838	3257	630	29.2	29.7	27.1	50.3	87.3	18.6
	Near- IR	721	722	692	2124	3688	2315	82.2	66.2	208.3	20.6	44.5	8.9
Moringa oleifera	Red	685	686	683	4389	1479	1775	29.4	29.6	28.9	119.1	39.8	49.0
	Near- IR	720	727	—	4494	3040	1781	72.4	81.1	113.5	49.5	29.9	12.5
Azadiracha indica	Red	685	686	682	3099	4322	1330	29.8	31.2	38.6	83.0	110.7	27.5
	Near- IR	724	730	747	2582	2560	258	61.6	49.6	48.2	33.4	41.2	4.3

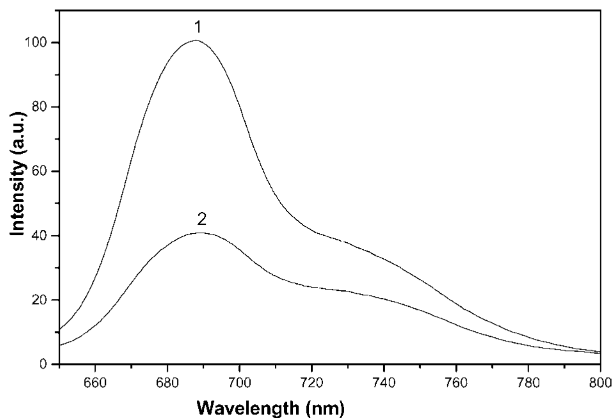

Fig. 5. Blue light (404 nm) excited chlorophyll emission spectra of lower (1) and upper (2) side of *Azadiracha indica* light-green leaf in the steady state conditions.

to the 682 nm peak. This is due together to the very lower chlorophyll content in the yellowish leaves and in the fact that the chlorophyll fluorescence spectra is no longer influenced by reabsorption of the chlorophyll fluorescence.

The increasing chlorophyll content in greening leaves generally induces a shift in the wavelength position of the maximum (Tables I and II). There is about 2 nm shift in the wavelength position of the maximum in the fluorescence spectra for the Light and fully-green upper leaf half and 1 nm in the corresponding lower leaf half. Instead in almost yellow leaves the shift is quite important (between 725 and 745 nm); in some case there is no shoulder due to the drastic reduction of the chlorophyll content (see, Fig. 4).

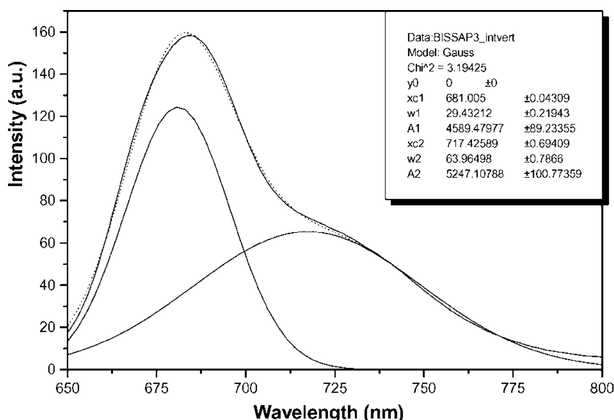

Fig. 6. Gaussian fit of upper leaf side chlorophyll fluorescence emission spectrum of *Hibiscus sabdariffa* light-green leaf excited at 404 nm.

Depending on the intensity of the incident light during growth and development of the plant, various responses including light adaptation of the photosynthetic apparatus and chloroplast ultrastructure can take place by the formation of two structurally different chloroplast: the shade-type and sun-type chloroplasts [5]. The latter possess a lower cross-section for light absorption (less light-harvesting chlorophyll proteins) and higher rates of photosynthetic quantum conversion than shade-type chloroplasts. In addition theses plants possess bifacial leaves (C_3 plants), i.e., a different structure and cell arrangement for the upper and lower leaf sides. The upper leaf half consist of densely packed and long palisade parenchyma which also contain higher chloroplast numbers and it therefore possesses a much higher chlorophyll content and density than the lower leaf half [5]. This will result in a stronger fluorescence for the lower side compared with the upper side (Fig. 5).

To find the exact peak position, the peak amplitude, the band width and the band area, the measured fluorescence emission spectra are analyzed by fitting the curves with Gaussian spectral functions. It was observed that curve fitting by using Gaussian peaks usually fits well the Chlorophyll fluorescence spectra [18–20]. The Gaussian spectra of *Azadirachta indica* yellow-green leaf resulting from the curve fitting analysis is reported in Fig. 6 (dotted lines). Tables I and II give the curve fitting parameters such as the peak center, full width at half maximum (FWHM) and the area under each Gaussian curve for different leaves of each species.

The absolute emission signal of leaves can vary from sample to sample due to small differences such as the excitation and sensing angles of the fluorescence, and the roughness and scattering properties of the leaf surface. Thus, the absolute fluorescence usually varies to a large degree than the fluorescence ratio. The fluorescence ratio, in turn exhibit much lower variation from leaf to leaf, represent reliable and reproducible means for the quantification of changes in the fluorescence characteristics of leaves and the LICF spectra can be analyzed by Fluorescence Ratio F690/F735 obtained from curve-fitted parameters. The FR F690/F735 at the maxima near 690 nm and 735 nm were calculated from the spectral intensity of the measured spectra and also from the amplitudes and areas of the Gaussian's curves and are showed in the Tables III and IV. The spectral intensities are measured at the peak position of the Gaussian curve.

As one can be seen in Fig. 7, the F690/F735 values show marked differences between that calculated from the Gaussian's curves amplitudes and those obtained from the Gaussian's curves areas. In any case Fluorescence Ratio has the same profile. The commonly used parameter is

Table III. The Upper Leaf Side Fluorescence Ratio F690/F735 Calculated From the Spectral Intensity of the Measured Spectra and also From the Amplitudes and Areas of the Gaussian's Curves

	Spectral intensity			Peak amplitude			Peak area		
Ratio F690/F730	L-G	D-G	Y-G	L-G	D-G	Y-G	L-G	D-G	Y-G
Adansonia digitata	1.58	1.71	3.56	1.06	1.24	2.84	0.35	0.44	0.26
Hibiscus sabdariffa	2.54	2.17	2.90	2.35	1.96	2.40	0.99	0.93	0.40
Moringa oleifera	2.00	1.44	3.09	1.56	0.87	2.02	0.57	0.30	0.26
Azadirachta indica	1.69	2.00	1.90	1.18	2.17	0.77	0.48	1.40	5.38

the Fluorescence Intensity Ratio (FIR) which refers to the calculated ratio from the intensities (either from the spectral intensities or the Gaussian's curves amplitudes).

The lower chlorophyll content will increase the fluorescence intensity compared to normal green leaves. Since the increase in the 690 nm region is much higher than the increase in the 735 nm region, lower chlorophyll content will increase the values of the ratio F690/F735. With increasing chlorophyll content in light-green leaves, the ratio F690/F735 decreases correspondingly from value of 1.5 to 1.2 and 0.9. In yellowish leaves with very-low chlorophyll content, the ratio F690/F735 exhibits high values of 2.5 to 8. Fully developed leaves which possess full photosynthetic function are characterized by low values for the ratio F690/F735 of approximately 0.8 to 1.1 for the upper leaf side.

An increased ratio F690/F735 is not only indicative of lower chlorophyll content; the values also increase when the process of photosynthetic electron conversion is affected and decline. Under many stress conditions which last for a longer time, the chlorophyll content peer leaf area unit is lower than in normal green leaves. This can be due to a decrease rate of chlorophyll accumulation during the leaf development as under mineral or water stress or to a partial loss of chlorophyll as in high light and heat stresses.

The chlorophyll content of the lower leaf half as well as the probability of the reabsorption of emitted chlorophyll fluorescence hence are much lower than in the upper leaf half. Consequently the fluorescence spectra at the lower leaf half is higher, particularly in the 686 nm region, than in the upper leaf half which makes the ratio F690/F735 higher in the lower than in the upper leaf half.

CONCLUSION

Under longer-lasting stress conditions, the plants are characterized mostly by lower chlorophyll content per leaf area unit and also by the decline of the rate of photosynthesis compared to plants growing under better and more normal environmental conditions. Although one always needs to know if in a stressed plant the fluorescence bands specially the red and far-red increase or decrease in the mean as compared to a control, one should never rely on the increase or decrease of the fluorescence intensity alone. One cannot identify a stressor by fluorescence measurements alone, one can however; considerably reduce the number of possible stress constraints to a few.

The fluorescence ratios proved to be very early stress and strain indicators, as they change with strain and stress begin and long before damage of the photosynthetic apparatus is detectable.

The results obtained with the blue excitation, provide new data as fluorescence signature and corresponding calibration of the fluorescence intensity ratio F690/F735 as indicators of the plant physiological state.

The physiological information obtained about plant health from fluorescence measurements must be supplied

Table IV. The Lower Leaf Side Fluorescence Ratio F690/F735 Calculated From the Spectral Intensity of the Measured Spectra and also From the Amplitudes and Areas of the Gaussian's Curves

	Spectral intensity			Peak amplitude			Peak area		
Ratio F690/F730	L-G	D-G	Y-G	L-G	D-G	Y-G	L-G	D-G	Y-G
Adansonia digitata	1.99	1.84	4.61	1.66	1.38	4.01	0.69	0.56	0.58
Hibiscus sabdariffa	2.54	2.17	2.90	2.44	1.96	2.09	0.87	0.88	0.27
Moringa oleifera	2.58	1.78	1.58	2.41	1.33	3.91	0.98	0.49	1.00
Azadirachta indica	2.61	2.56	6.07	2.48	2.69	6.44	1.20	1.69	5.16

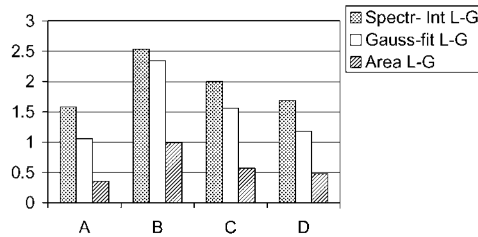

Fig. 7. The Fluorescence ratio F690/F735 diagram at the maxima near 690 nm and 735 nm calculated from the spectral intensity (grey) of the measured spectra and also from the amplitudes (white) and areas of the Gaussian's curves. (A) *Adansonia digitata*, (B) *Hibiscus sabdariffa*, (C) *Moringa oleifera*, and (D) *Azadiracha indica*.

by compliments information obtained from passive reflectance and absorbance measurement.

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