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CHLOROPHYLL FLUORESCENCE AS INDICATOR OF LEAF SENESCENCE IN *SITAW* PLANTS

by

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ABSTRACT

An initial study on the use of fluorescence spectra as indicators of some plant characteristics was done. The excitation was provided by a 75-watt pulsed xenon lamp.

Bush sitaw leaves ranging in age from 7 days to 42 days, were used as samples in the experiment. This range of ages covered the extreme cases of young shoots to those starting to manifest chlorosis. The varying amount of chlorophyll in leaves of differing ages is manifested as variations in the fluorescence spectra. The known fluorescence of chlorophyll in the wavelength region between 650 to 750 nm was observed, and changes in the peak intensity at 680 nm and in the total emitted intensity were noted as indicators of leaf age. The length of time leaf samples of the same age were set aside as cut foliage prior to spectral measurements also showed effects on the fluorescence spectra.

The paper suggests extensions of this study, using UV or dye laser excitation, for further applications in the assessment of plant condition, with the hope of supplementing established techniques used by botanists. Such extensions include *in vivo* measurements of time-averaged fluorescence spectra, and with the proper excitation pulse duration, time-resolved fluorescence decay measurements.

1. Introduction

Fluorescence is a process in which radiation is emitted by molecules or atoms that have been raised to an excited state by absorption of radiation. Specifically, fluorescence is restricted to those emission processes which originate and terminate in states of the same multiplicity, i.e. singlet-singlet transitions[1].

From the time G.C. Stokes made his first fluorescence research in 1852[2] up to the present-day studies in laser-induced fluorescence[3]-[9], hundreds of researches involving fluorescence spectroscopy and development of fluorescence instrumenta-

tion had been undertaken. Reports on fluorescence emissions of organic matter and other materials have appeared in various books, journals, and magazines. This paper presents a study on the fluorescence of one type of organic molecule, chlorophyll, insofar as it indicates leaf aging and senescence.

Chlorophyll has long been known to be a fluorescent molecule, and because of its important role in photosynthesis, is well-studied, using both *in vitro* and *in vivo* techniques. *In vitro* studies involve use of purified chlorophyll samples, and as such require some tedious and lengthy extraction procedure, aside from having to contend with solvent effects. However, all other pigments occurring in plants which are also fluorescent are excluded, and therefore the observed emission spectra will definitely be that of chlorophyll alone. *In vivo* methods using fresh functioning leaves, whether they be excised from, or still attached to the living plant, are more convenient as far as the sample preparation phase is concerned, but then the expected signal-to-noise ratio in the fluorescence measurement is a lot poorer than that for *in vitro* techniques. Thus, more sophisticated light sources and detection methods are required. The main attraction of *in vivo* techniques is its non-destructive and non-invasive nature, which allows real-time investigation of the photosynthetic process, using appropriately short light pulses from laser sources.

Early studies on chlorophyll have established two forms, labelled chlorophyll a and chlorophyll b, which have slightly different molecular structures. Chlorophyll a has an emission band at 685m 2 nm, and a satellite peak at around 740 nm. Chlorophyll b has a fluorescence peak at around 720 nm. Excitation occurs in the blue-violet (Soret) band, leading to the second electronic excited state, followed by non-radiative transition to the lowest excited state[10].

There are numerous reports on the use of fluorescence technique for investigating plant conditions. Reports on the fluorescence properties of pigments within the chloroplasts, fluorescence characteristics of plant cells subjected to various growth conditions such as exposure to varying light sources and intensities, low temperatures, and even plant poisons such as DCMU, have been published [4]-[6], [11]-[15]. Results of these studies have shed light on the correlation between observed fluorescence emission and the various conditions that the fluorescing samples are in. Quantum yields of fluorescence of several pigments in plants, as well as excitation lifetimes, were determined way back in the 50's [1], [14], [15]. The effect of pre-illumination using different light sources on the intensity of the fluorescence emission and its spectral profile has also been studied [16].

Researchers have gained a deeper understanding of what components are and what processes go on inside a plant cell through the study of chlorophyll fluorescence. It has been found that the primary pigment involved in the conversion of light energy to chemical energy during photosynthesis is chlorophyll a [17]-[19]; that the concentration of these primary pigments in a photosynthetic unit affects the observed fluorescence [11]; that each type of chlorophyll and the other pigments have their own characteristic fluorescence as well as absorption bands which help in their identifica-

tion [11], [18]; and that fluorescence measurements serve as indicator of the efficiency of photosynthesis in plants, the amount of fluorescence being taken as wasted light [11], [14], [18].

Technological advancement in instrumentation, particularly in the production of better light sources such as lasers, and detectors such as photon counting systems, permitted better signal-to-noise ratio, especially for in vivo studies. The pioneering work of Hickman on laser-induced fluorescence (LIF) of marine plants has been cited in a number of references [4]-[7]. The groups of Chappelle [4]-[7] and Macfarlane [9] have used LIF as a means of detecting plant stress and of identifying the plant type or plant species. Chappelle's findings revealed that water stress and mineral deprivation resulted in changes in the fluorescence intensities of soybean and corn, and that different plant types could be identified by the relative intensities of the different fluorescence peaks, or by the number of fluorescence bands of the samples.

Loaded on an aircraft, the LIF system could also be used for remote sensing of vegetation characteristics, assessment of plant vigor, identification of plant types, and estimation of timber yield over a large forested area [4], [7], [20].

In this study, the fluorescence of chlorophyll is measured and investigated for its correlation with the age of a leaf. Relating the age of a leaf to its chlorophyll fluorescence profile may not have economic value per se, but the main objective here is to learn and understand the technique of fluorescence spectroscopy, preparatory to designing a set-up for quick, easy, non-invasive, and non-destructive assessment of plant conditions, and the effects of various stresses such as dehydration and light deprivation, and pesticide uptake in plants.

Thus, this study is to be regarded as exploratory in nature, with the results taken as mere tests for the validity of the fluorescence technique, and an indicator of its sensitivity limits, given a specific excitation system and detector.

II. The Experiment

II.1. *Instrumentation*

A schematic diagram of the measurement set-up is shown in Figure 1. The main components of the system are the excitation lamp, the sample, the spectrometer, and the detector.

The excitation source used in the experiment is a 75-watt xenon stroboscopic lamp (Cenco) run at a repetition rate of 708 kHz. Xe has a broadband emission in the UV and visible bands, from 270 nm to 700 nm, which matches very well the absorption bands of chlorophyll. The light from the Xe lamp was focused by a quartz lens of diameter 50 mm and focal length 10 cm onto a quartz cell containing the leaf samples soaked in methanol. To minimize stray light, a black cardboard tube was used, extending from the lamp to the focusing lens. Other black cardboard shields were used wherever necessary to prevent scattered light from entering the entrance slit of the spectrometer.

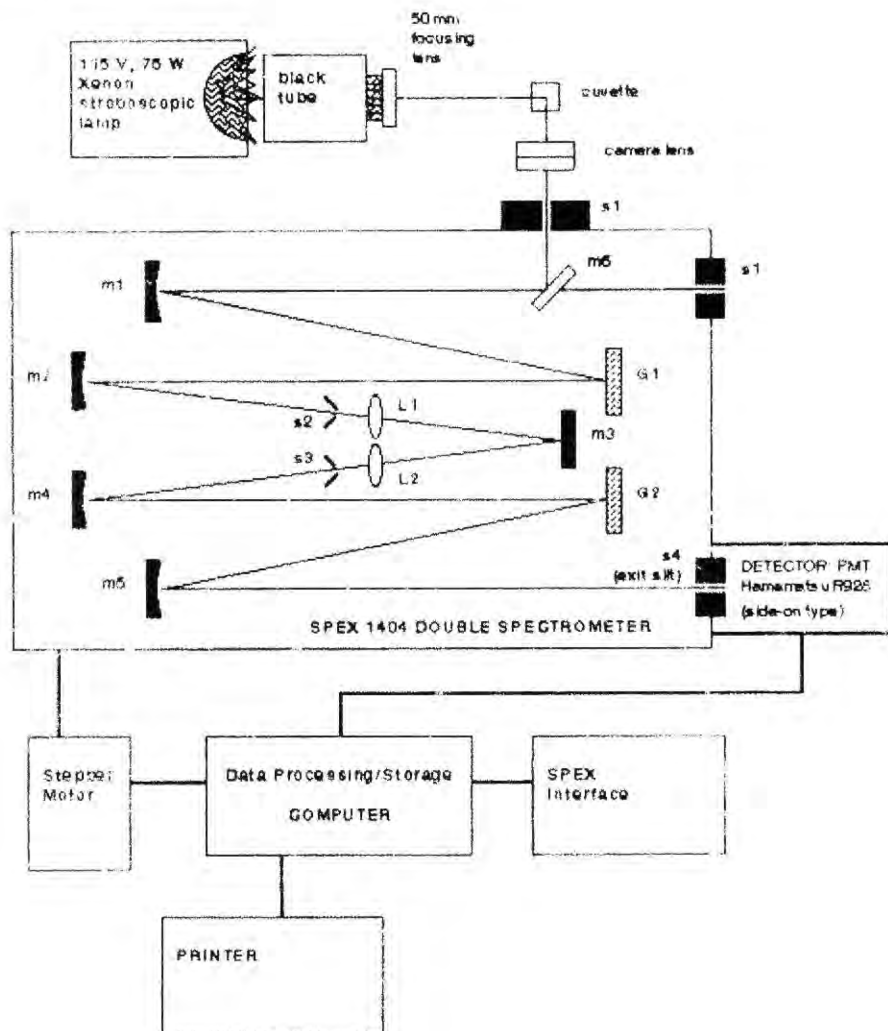


Figure 1. Schematic diagram of measurement set-up for this experiment.

The sample cell has dimensions of 1 mm x 1 mm x 10 mm (Sargent-Welch), oriented such that one face was centrally illuminated by the Xe lamp. The fluroescence emission from the sample was collected at right angles to the Xe illumination by a 50 mm camera lens (Canon) adjusted from maximum readings in the spectrometer output.

The spectrometer used in the experiment is a double-grating model with f= 0.85 m and aperture ratio f/7.8 (Spex 1401) with a spectral resolution of 0.05 nm, and

equipped with a stepping motor drive. The two gratings are holographic, with 1800 grooves/cm, and a dispersion of 0.275 mm per mm at 514.5 nm. The spectrometer is also equipped with a fully computer-interfaced data acquisition module, and a photomultiplier tube (Hamamatsu R928) as detector. The slit settings used during the experiment are as follows: entrance slit and exit slit - 1500 μm; middle slits - 2500 μm. These slit settings were chosen because they are wide enough to collect fluorescence emission for a good signal-to-noise ratio, and still give the resolution needed to separate the 680 nm and 740 nm peaks. Scanning over the wavelength range of 600 nm to 800 nm was done at increments of 1 nm, with an integration time of 5ms for each step. Bias voltage of the PMT was set at 800 volts.

The detector is attached directly to the exit port of the spectrometer by a photomultiplier housing. It has an extended red, high sensitivity, multialkali photocathode, with range of spectral response from .185 nm to 900 nm, peaking at 400 nm. Specifications supplied by the manufacturer indicate a sensitivity of 40 mA/W at 650 nm, dropping to about 25 mA/W at 750 nm.

Software (DM 3000R) provided by the spectrometer manufacturer was a means of quickly processing, viewing, and printing the fluorescence data. Averaging, smoothing, and integration procedures were all done using the math functions provided in the software.

II.2 *Test Runs.*

Prior to the measurement of chlorophyll fluorescence spectra from the various leaf samples, test runs were taken to check the calibration of the spectrometer, and to estimate the expected range of intensities that could be detected by the measurement system. Background noise from stray light and from the detection system was also measured.

The spectrum of the Xe light scattered by the methanol-filled quartz cell was taken, as well as the spectrum of Xe alone. These were stored in the computer for appropriate correction of the measured chlorphyll fluorescence spectra later on. No contributions to the background spectrum were discerned from methanol and quartz. Figure 2 shows the spectrum of Xe over the range of wavelengths 600 nm to 800 nm, which includes the chlorophyll fluorescence band from 650 nm to 750 nm. Except for some spikes which are readily identifiable, the Xe spectrum is nearly flat over this range.

To protect the photomultiplier tube from saturation while taking the Xe spectrum with the wide slit openings of 1500 μm, neutral density filters constructed out of x-ray negatives were used as attenuators.

II.3. *The Samples and Sample Preparation Procedure*

Each of the plant samples were grown until each had a set of twin leaves and two sets of trifoliates, making a total of eight leaves per sample. Sample used were *bush sitaw* (*Vigna sesquipedalis*) grown in normal environment, meaning suffi-

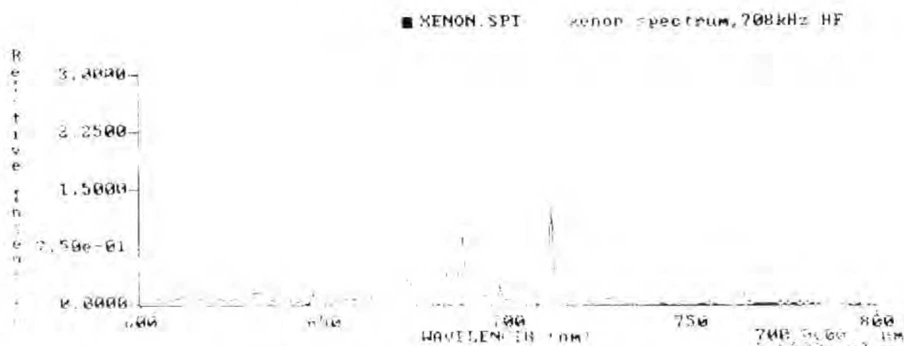


Figure 2. Spectrum of Xenon over the wavelength range of 600 nm to 800 nm.

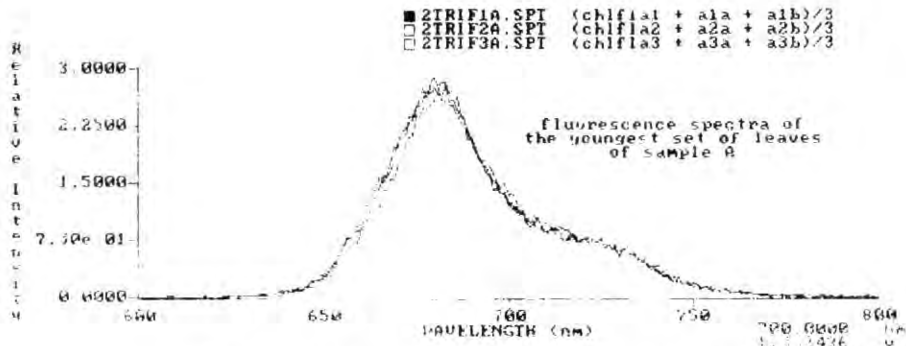
cient light, water and ventilation, from germination to the day of the experiment. These samples were planted in cardboard boxes filled with the same type of soil for all samples.

Two groups of five plants each were employed in the experiment. The first set labeled A to E all had 7-day-old second trifoliates, 18-day-old first trifoliates, and 28-day-old twin leaves. The second set labeled F to I had 21-day-old second trifoliates, 33-day-old first trifoliates, and 42-day-old twin leaves. The fluorescence spectra of all the leaves from the second set were taken two weeks after the first set. Ages of the leaves ranged from the youngest of 7 days, to the oldest of 42 days, at which time some chlorosis was beginning to be apparent.

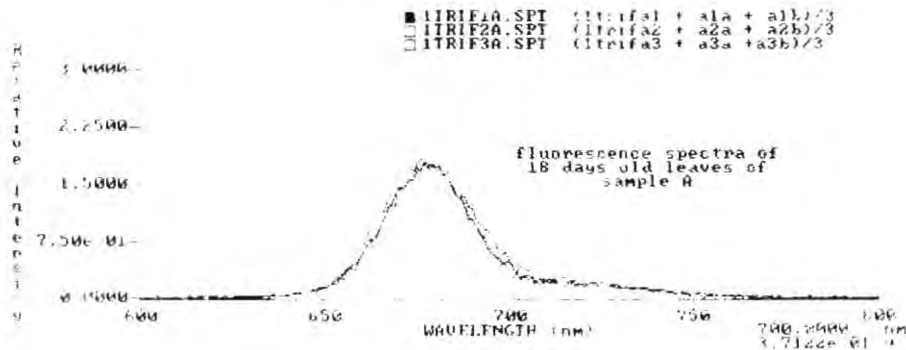
Leaves of the same age were excised at the same time, rinsed with tap water and dried with unscented tissue paper. After rinsing and drying, each leaf was prepared for extraction. In order to maintain a constant leaf area in all of the samples throughout the experiment, 17 holes were punched through each leaf, using a standard single-hole paper puncher. This ensured that the total leaf area for all the samples was constant at 333.88 mm^2. This requirement of 17 leaf disks from each sample presented a limitation to the minimum age of leaf that could be studied. The minimum age that could be included in this experiment was 7 days, because at this time, the leaves had grown to a size large enough for 17 holes to be punched, and also they were firm enough to allow neat punching. The leaf disks were gathered and put in a quartz sample cell containing 3.5 ml of methanol, and allowed to stand for about 1.5 to 2.5 hours before the fluorescence spectra were taken. Methanol was used as solvent because of the expected high quantum yield of chlorophyll in methanol [14].

Sample preparation was done under minimal lighting conditions because chlorophyll undergoes degradation with exposure to light.

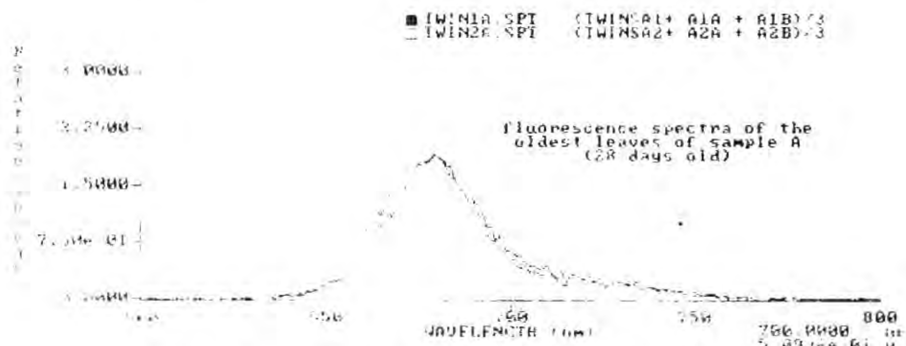
No purification processes were done in extracting the chlorophyll pigment as the objective of the experiment was to investigate the properties of samples whose constituents are, as much as possible, of the same kind as those in live plants.



3.a. Fluorescence emission spectra for 7-day-old leaves



3.b. Fluorescence emission spectra for 18-day-old leaves.



3.c. Fluorescence emission spectra for 28-day-old leaves.

Figure 3. Set of spectra for leaves of varying ages, sample A. Note reproducibility of obtained spectra for each set of leaves.

III. Results and Discussion

III.1 *Chlorophyll Fluorescence as an Indicator of Leaf Age.*

A set of sample spectra for eight leaves belonging to one plant (labelled as plant A, from the first group) is shown in Figure 3. The first group of spectra (3.a) shows the emission spectra for the second trifoliates, the youngest leaves at 7 days. The second group (3.b) shows the spectra for the first trifoliates at 18 days old, while the third group (3.c) shows the spectra for the twin leaves at 28 days old. Another set of sample spectra as shown in Figure 4 was taken from one of the samples belonging to the second group. In this case the youngest leaves were 21 days old and the oldest leaves were 42 days old. Clearly, the youngest leaves at 7 days old had the highest peak intensities around the wavelength of 680 nm, and the oldest leaves at 42 days had the weakest fluorescence emission. However, the fall in fluorescence intensity is not monotonic, as can be seen from Figure 5, which plots the total fluorescence intensity vs. age. One can discern a temporary drop in the emission between 7 and 17 days, and then a slow increase between 17 and 27 days, a levelling-off between 27 and 37 days. As senescence sets in, there is again a decrease in the total fluorescence intensity, during the period 37 to 42 days. At 42 days, chlorosis is beginning to be apparent, and in some cases, may even be in a late stage already.

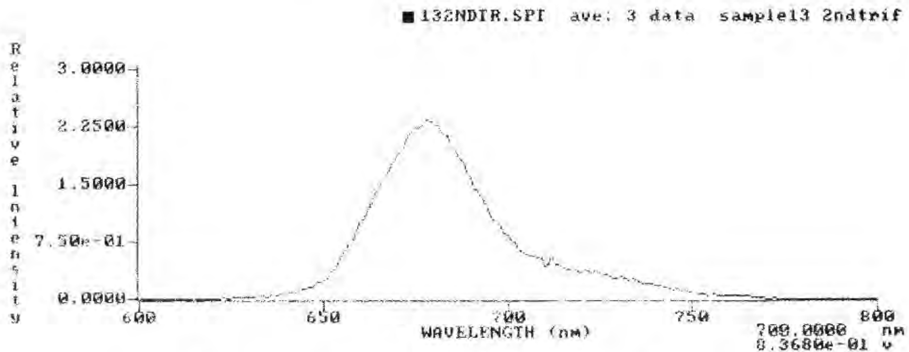
Figure 3 shows that for each group of the same age, and coming from the same plant sample, the fluorescence spectra are highly reproducible in terms of shape and maxima. This reproducibility gives assurance that the observed changes in the spectra may indeed be attributed to variations in the parameter whose effect is being studied, in this case, the age of the leaf.

Furthermore, in a majority of samples, it could be observed that as the leaves increase in age, the 720-740 nm and decreases in intensity or flattens out. For the youngest set of leaves, the 720-740 nm hump is obvious, but this is diminished in the 28-day-old leaves. This hump is nearly totally flattened out for chlorotic 42-day-old leaves. The ratio of the relative intensity peaks at 680 nm and 740 nm is also sometimes used as measure of the changes in fluorescence spectral profiles. However, in this study, since no correction for detector response nor spectrometer grating efficiency was made, this ratio could not be used as a measure of spectral change.

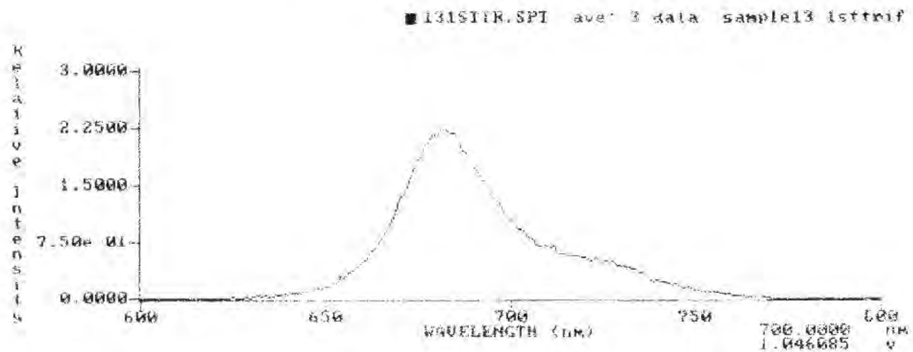
While only the results for sample A and sample 1 have been shown here as representative results, the spectral features described are also observed in the other samples.

III.2. *The Effect of Chlorosis.*

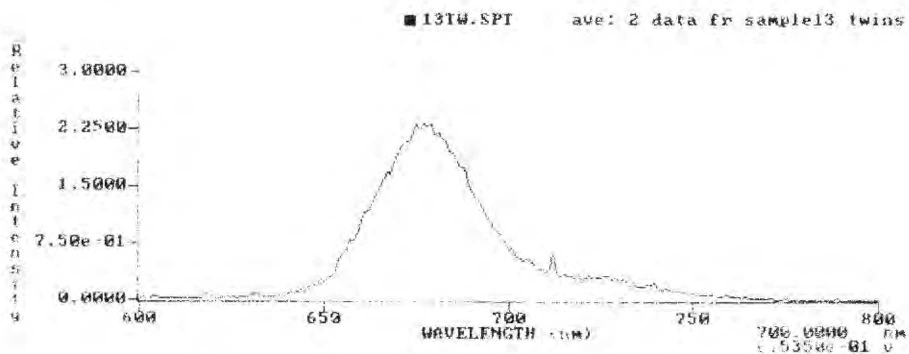
Chlorosis is that stage in plants wherein the leaves lose their green color due to old age or environmental stress such as insect infestation, nutrient deficiencies, dehydration, extreme changes in temperature, and soil conditions [4]. A comparison between the fluorescence spectra of the youngest leaves and the old,



4.a. Fluorescence emission spectra for 21-day old leaves.



4b. Fluorescence emission spectra for 32 day-old leaves.



4c. Fluorescence emission spectra for 42-day old leaves.

Figure 4. Set of spectra for leaves of varying ages, sample I.

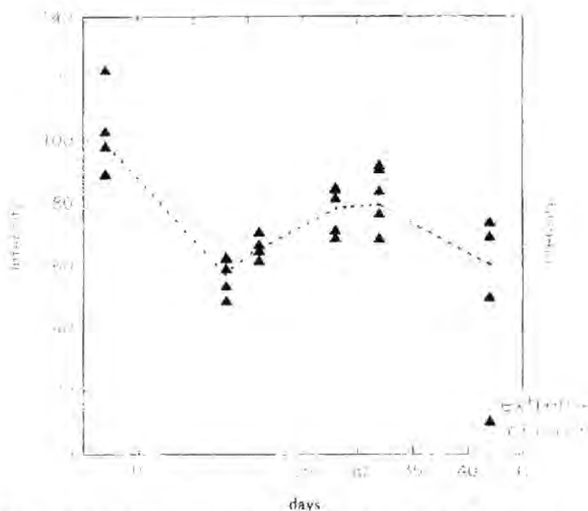


Figure 5. Plot of total fluorescence intensity vs. age of leaves.

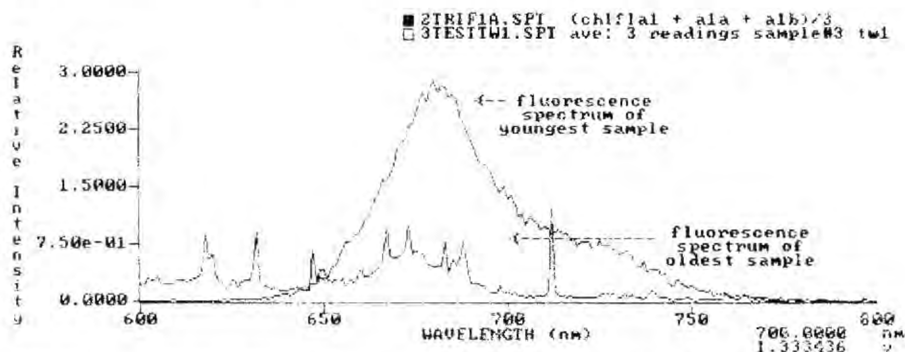


Figure 6. Fluorescence emission spectra for youngest leaf and most chlorotic leaf.

chlorotic leaves showed a considerable decrease in the fluorescence intensity of the older leaves. The degree of degradation of the emission spectra depended on which stage of chlorosis the leaves were in Figure 6 shows the fluorescence spectrum of a representative of the youngest samples and one of the oldest leaves in the late stage of chlorosis. A major part of the old leaf was already yellowed and brown at the edges. The youngest leaf, on the other hand, was still very green in color and showed no sign of infestation. Figure 7 shows another set of spectra obtained for youngest and oldest leaves. In this case the old leaf was just at the early stage of chlorosis, manifesting just a few yellow spots. The fluorescence intensity of the old leaf did not decrease as much as the old leaf in Figure 6. But in both cases illustrated in Figures 6 and 7, the fluorescence intensity of the chlorotic

leaves decreased so much that the background Xe spectrum is already noticeable, judging from the presence of the spikes. A corrected version of Figure 6 is shown in Figure 8. Figure 9 shows the integrated spectral profiles corresponding to the pairs of spectra shown in Figure 6. Again, the large amount of decrease in total fluorescence intensity can be readily seen.

III.3. *Effect of Lengthened Exposure as Cut Foliage.*

An additional observation, not originally intended as part of this study, was made during the experiment. This was the effect of lengthened exposure of the leaves as cut foliage, an unavoidable condition as the sample preparation procedures for each leaf took a considerable amount of time. Notwithstanding the fact that precaution was taken to keep the leaves in the dark while they were waiting for processing, and to do the spectral measurement runs at night, some degradation of chlorophyll took place, as manifested in the drop in intensity with longer exposure time.

During the experiments, the leaves were cut by groups according to age. The youngest leaves, for example, were cut from all the five *bush sitaw* plants belonging to the first group. While preparing these samples one by one some leaves were set aside in the dark for some time before they were punched and soaked in methanol. Graphing the peak relative intensity of these groups against the length of time they were set aside as cut foliage showed some decrease of about 21% over a period of 820 minutes, as shown in Figure 10a-10c. A sample of the actual spectra for 4 sets of 7-day old leaves is shown in Figure 11.

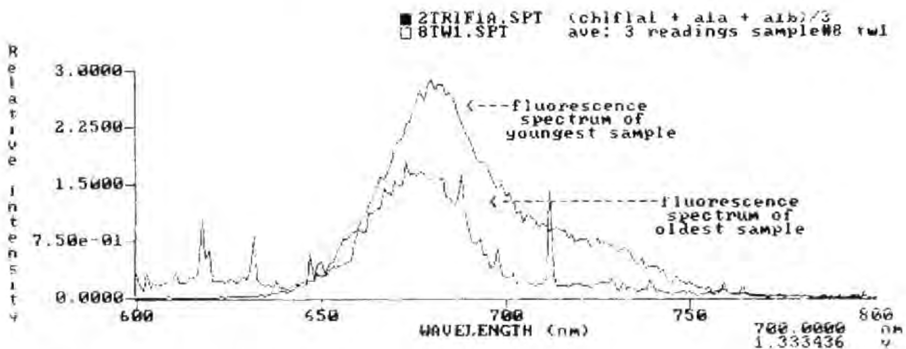


Figure 7. Another set of fluorescence emission spectra for youngest leaf and for old leaf just beginning to manifest chlorosis. Compare with Figure 6.

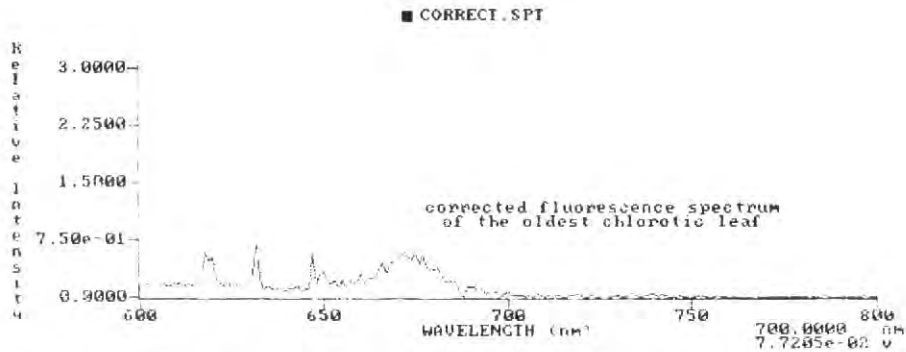


Figure 8. Emission spectrum of most chlorotic leaf, corrected for background Xe spectrum.

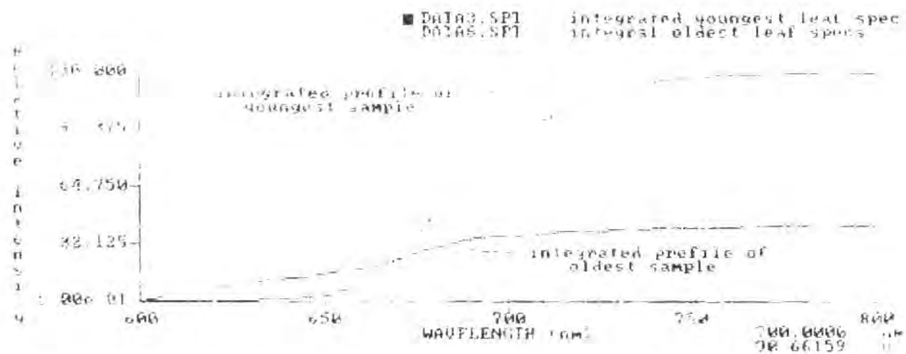
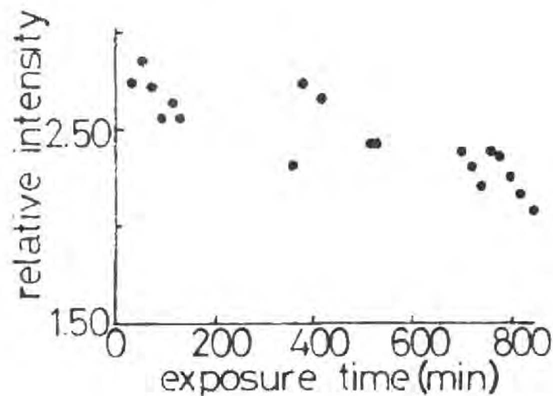
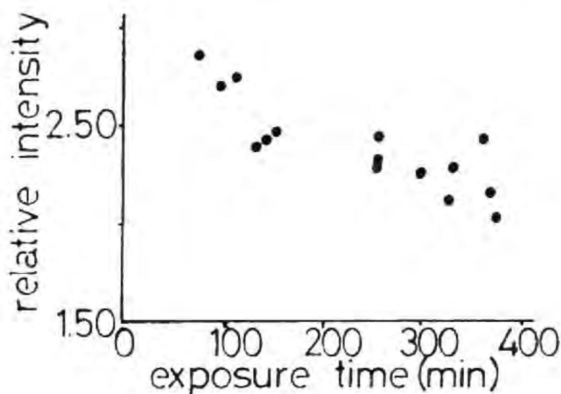


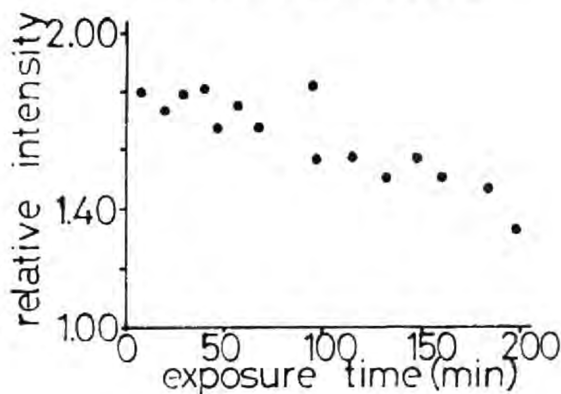
Figure 9. Integrated spectral profile for the pair of spectra shown in Figure 5.



10.a. Data for 7-day-old leaves.



10.b. Data for 18-day-old leaves



10.c. Data for 28-day-old leaves.

Figure 10. Drop in fluorescence peak intensity with length of exposure time as cut foliage.

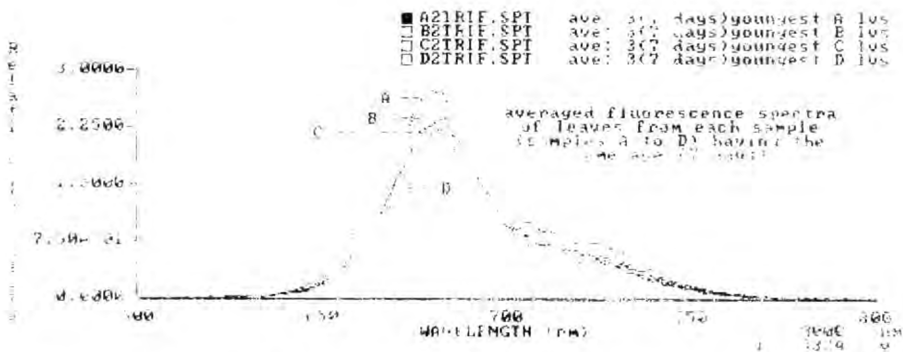


Figure 11. Spectra for leaves of the same age (7 days) but varying exposure time as cut foliage.

IV. Conclusions and Recommendations

IV.1. *Conclusions*

Various changes in total fluorescence intensity were observed for leaves of *Vigna sesquipedalis* whose ages ranged from 7 to 42 days. An initial drop in the total fluorescence intensity was observed, followed by a slow rise as the leaves matured, then a leveling off, and again a decrease as the leaves became senescent. Since growth conditions were normal and no insect infestation nor disease were apparent, these changes in fluorescence intensity are attributed to the changes in the amount of chlorophyll in the leaf. However, whether this observed trend can be correlated to the growth and maturation processes of a plant is beyond the scope of this work, whose main objective is to focus on the technique of fluorescence spectroscopy, and its applicability to the observation of some plant characteristics.

The reproducibility of the spectra for samples of the same age and coming from the same plant gives an assurance that the observed changes in the fluorescence spectra may indeed be attributed to variations in leaf age, the parameter being studied here. Since chlorophyll is a molecule that degrades with exposure to light, some effect of the length of time the leaves were exposed as cut foliage prior to sample processing procedures was also noted on the fluorescence spectra. This problem can be easily avoided by using *in vivo* techniques of fluorescence spectroscopy.

This paper is a preliminary investigative work on the applicability of using fluorescence techniques in studying some characteristics of plants, and the results, though qualitative in nature, have shown that fluorescence methods can indeed be used to detect changes in plant conditions.

IV.2. *Recommendations*

Since *in vitro* techniques are tedious and may even present problems related to degradation of chlorophyll with exposure to light, *in vivo* technique are recommended for any follow-up to this project. The latter method has the advantage of having no sample preparation requirements, except perhaps for wiping clean the surface of living, functioning leaves before exposure to the excitation light. Being non-destructive and non-invasive, *in vivo* techniques are ideal for real-time spectroscopy of processes inside a living leaf, assuming that appropriately short laser light pulses of the correct wavelength are available.

Instead of using a non-coherent excitation light such as the Xe source that was used in this study, coherent sources such as lasers would be more advisable for *in vivo* techniques. The nitrogen laser, if properly shielded for elimination of electromagnetic interference effects, can be a cheap source of excitation light for future studies in laser-induced fluorescence of green plants. However, for real-time spectroscopy, ultrashort light pulses from wavelength-tunable dye lasers are required.

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