Fluorescence: A Diagnostic Tool for the Detection of Stress in Plants

Emmett W. Chappelle[1], Lawrence A. Corp[2], James E. McMurtrey[3],
Moon S. Kim[2], and Craig S.T. Daughtry[3]

[1]Laboratory for Terrestrial Physics
NASA/GSFC, Greenbelt MD 20771.

[2]Science Systems and Application Inc.
5900 Princess Garden Pkwy., Lanham, MD 20706.

[3]Remote Sensing Research Laboratory
ARS, United States Department of Agriculture
Beltsville, MD 20705.

ABSTRACT

Green vegetation when excited by specific wavelengths of light dissipates a portion of the absorbed energy as light emissions in the form of fluorescence in several broad areas of the spectrum. Currently, leaf level fluorescence emissions have been broken down into five primary regions, namely; ultraviolet (UV), blue, green, red, and near-infrared (NIR). The optimal excitation wavelengths for each of these bands was verified for healthy soybean leaves through the use of the EEM (excitation and emission matrix). Intact vegetation when excited at 280 nm emits substantial fluorescence in two bands; the first centered near 335 nm (UV band), and the second centered near 440 nm (blue band). UV band fluorescence from vegetation treated with varying levels of nitrogen decreases relative to the blue fluorescence as a function of total protein concentration. These studies indicate that *in vivo* UV band fluorescence can be utilized as a non-destructive tool to remotely sense variations in protein concentration due to nitrogen fertilization level. It has been well established that this fluorescence emission originates from proteins containing aromatic amino acids. The majority of plant proteins contain these amino acids and as a result have the potential to fluoresce in the region of the spectrum discussed here. Pure ribulose 1,5-bisphosphate carboxylase (RubisCO) in aqueous solution exhibited intense UV fluorescence characteristics with excitation and emission distributions similar to those of intact vegetation. Due to its high concentration (up to 70 % of the soluble leaf proteins) we believe this protein contributes to the UV band fluorescence emanating from the intact leaf.

The red and NIR fluorescent emissions can be excited within the broad wavelength region from 250 to 675 nm with excitation maxima at 430 nm, 470 nm, 600 nm, and 660 nm. The ratio of red to NIR fluorescence excitation spectra produces a ratio spectrum which exhibits striking similarities to the action spectrum of photosynthesis. The relative differences between these two emission bands depend on the wavelength of excitation. Moreover, by comparing the ratio spectrum of a healthy versus nitrogen deficient leaf, one finds areas of crossover where trends can be completely reversed by changing excitation wavelength. As a result, the success of studies involving the measurement of chlorophyll *a* fluorescence depend greatly on the appropriate selection of excitation wavelength. Fluorescence sensing systems based on the above emission bands are being proposed or developed for ground based mobile vans, helicopters, and small aircraft. The goals of these efforts were to better define the origins of fluorescence and to improve our understanding of these light emissions in relationship to the physiological status of the plant.

Key Words: Fluorescence, Plant Stress, Remote Sensing, Nitrogen Fertilization Level, Remote Estimation of Protein Concentration.

1. INTRODUCTION

Vegetation, when exposed to long wave ultraviolet (UV) radiation of sufficient energy, dissipates a portion of the absorbed light energy as fluorescence with emission maxima near 450, 525, 685, and 740 nm[1]. The broad blue-green fluorescence band ranges from 360 to 650 nm with a principal maximum at 450 nm and a shoulder near 525 nm. The fluorescence spectra of a number of pure plant constituents were obtained, in order to assist in the identification of the compounds responsible for the fluorescent maxima[2,3,4,5]. Plants were extracted in both aqueous and organic media. Part of the dynamic portion of the blue band fluorescence has been attributed to the water soluble compound nicotinamide-adenine dinucleotide phosphate in the reduced form (NADPH) with the more static portion due to relatively inert structural compounds of the leaf epidermis and cell walls such as polyphenolics and lignin[2,4,5,9].

Strong candidates for the green band fluorescence encompass two broad categories of plant constituents, namely; flavins and carotenoids. Time-resolved fluorescence measurements provide strong evidence to support the green fluorescence emission by riboflavin and/or flavin mononucleotide phosphate[7,8]. To date the fluorescent properties of pure b-carotene have been highly debated with some taking the stand that the compound in its purist form does not fluoresce at all[4]. However, the fluorescent properties of the carotenoid accessory pigments as they function inside the light harvesting antenna complex of photosynthesis have yet to be determined.

A UV band of fluorescence with an emission maximum near 335 nm results when vegetation is excited with 280 nm radiation and is primarily due to proteins containing aromatic amino acids[10,11]. RubisCO, whose function is to catalyze CO_2 uptake in photosynthesis, constitutes up to 70% of the soluble plant proteins. This enzymatic plant protein exhibits fluorescence characteristics in accordance with a tryptophan containing protein and is believed to make a contribution to the UV band fluorescence. Furthermore, it has been observed that the UV band fluorescence emission from intact vegetation decreases with decreasing nitrogen fertilization[12].

Under optimal growth conditions the majority of light absorbed by plant chlorophylls and carotenoids is utilized in photosynthesis with less than 3% of the absorbed light energy being dissipated as fluorescence emissions near 685 (red) and 740 nm (NIR). The magnitude of these fluorescence emissions on exposure of a plant to light are governed by chlorophyll concentration and photosynthetic activity. The primary roles played by certain nutrients in photosynthesis and chlorophyll synthesis suggest that nutrient deficiencies could be detected on the basis of changes in these fluorescence emissions[1,2,15,16]. The red fluorescence emission has been attributed to chlorophylls associated with photosystem II (PS II) while the NIR fluorescence emission has been more closely associated with chlorophylls at or near the antenna of Photosystem I (PS I)[13]. The fluorescence ratio 685/740 nm could relate to the push pull nature of the two photosystems as well as an indicator of the distribution of light energy between the two photosystems[18]. In addition, several studies have demonstrated that linear and curvilinear relationships exist between certain ratios of the above fluorescence maxima to pigment concentrations and rates of photosynthesis[2,3,4,5,15].

2. METHODS AND MATERIALS

2.1. Preparation of Plant Material

Different nitrogen (N) levels were obtained by growing soybeans in perlite with a full complement of nutrients minus N. N was varied from 0.004 M to 0 M urea to produce levels of N deficiency. These levels correspond to 100%, 75%, 50%, 25%, and 0% of the N required for optimal growth. Nutrient solutions were applied once a week and the following measurements were made respectively on the uppermost fully expanded leaf after eight weeks of growth.

2.2. Rate of Photosynthesis

The rates of photosynthesis of soybeans were determined using an infrared gas analyzer (LI-COR 6200, LI-COR Inc., Lincoln NE) in the closed mode. The measurements were made in the laboratory with light source consisting of a combination of water-cooled low pressure sodium lamps and alkaline metal halide lamps providing an intensity of 1800 μmole m^{-2} s^{-1}. The ambient CO_2 concentration was maintained at 350 ppm. The temperature within the photosynthesis chamber did not exceed 27°C, while the relative humidity was maintained through desiccant at 38%.

2.3. Fluorescence Excitation and Emission Spectra

A spectrofluorometer (Fluorolog II, Spex Industries, Edison NJ) was used to collect fluorescence excitation and emission spectra. The spectrofluorometer utilized two 0.22m double monochrometers. The excitation monochrometer was attached to a 450 W xenon lamp which allowed variation of the excitation radiation. The emission monochrometer was attached to a photon-counting photomultiplier tube corrected to obtain linearity throughout the emission wavelength range of 290 to 850 nm while voltage readings were calibrated to photon counts per second (cps). Fluctuations in lamp intensity were corrected by using a beam splitter to deliver a portion of the excitation radiation to a rhodamine dye cuvette. The fluorescence response of rhodamine dye was monitored by a silicon photodiode. This response was used to generate correction factors for minimizing the effect of changes in lamp intensity as a function of wavelength. Excitation spectra were acquired by fixing the emission wavelength (usually at the fluorescence emission maximum) and recording emission intensities while stepping through a shorter wavelength region of the spectrum. Conversely, emission spectra were obtained by fixing the excitation wavelength and recording emission intensity while stepping through a longer wavelength region of the spectrum. Both excitation and emission spectra were acquired at a 1 mm slit width yielding 1.7 nm resolution. Leaf samples were held in place by an anodized aluminum solid sample holder.

2.4. Pigment and Protein Determinations

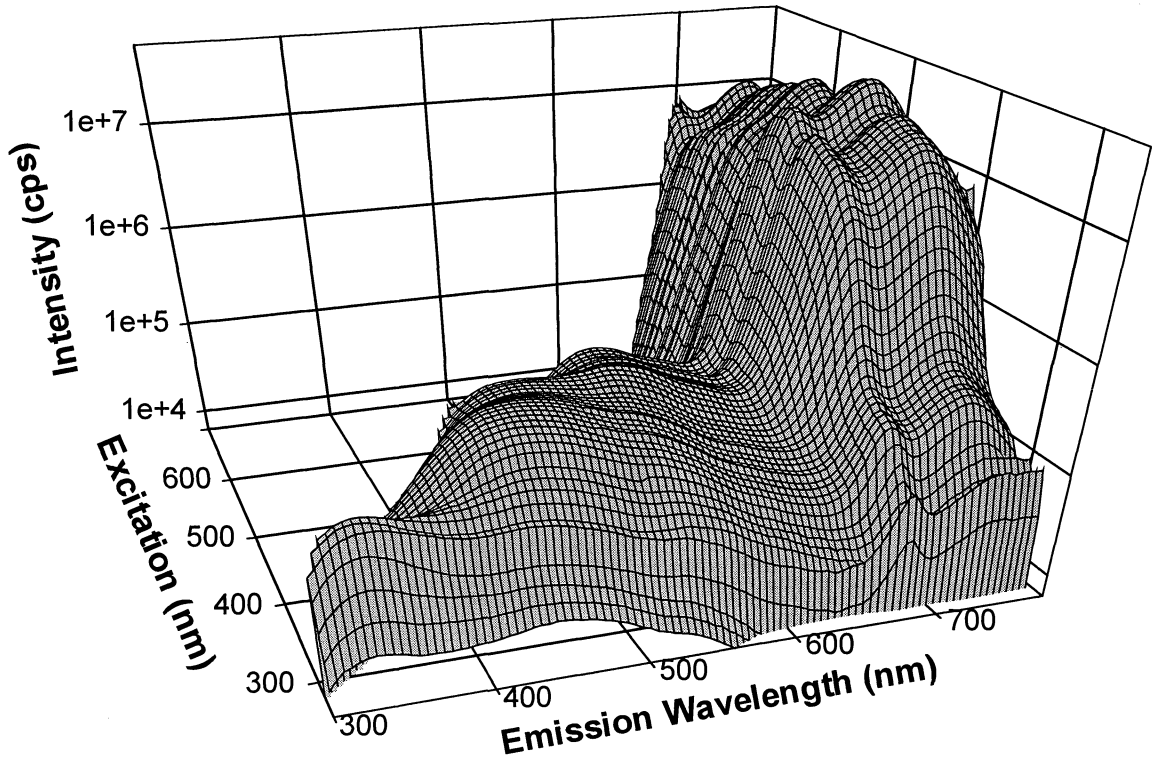
Leaf disks (2.54 cm^2) were extracted in dimethyl sulfoxide (DMSO) and absorption measurements were made using a Perkin-Elmer dual beam spectrophotometer. Pigment concentrations were determined by methods modified from Lictenthaler 1987. The remainder of the leaf was freeze dried at - 45 °C then ground into a fine powder. Total nitrogen was determined using Dumas combustion[20]. Total protein was estimated by multiplying total nitrogen by 6.25[21].

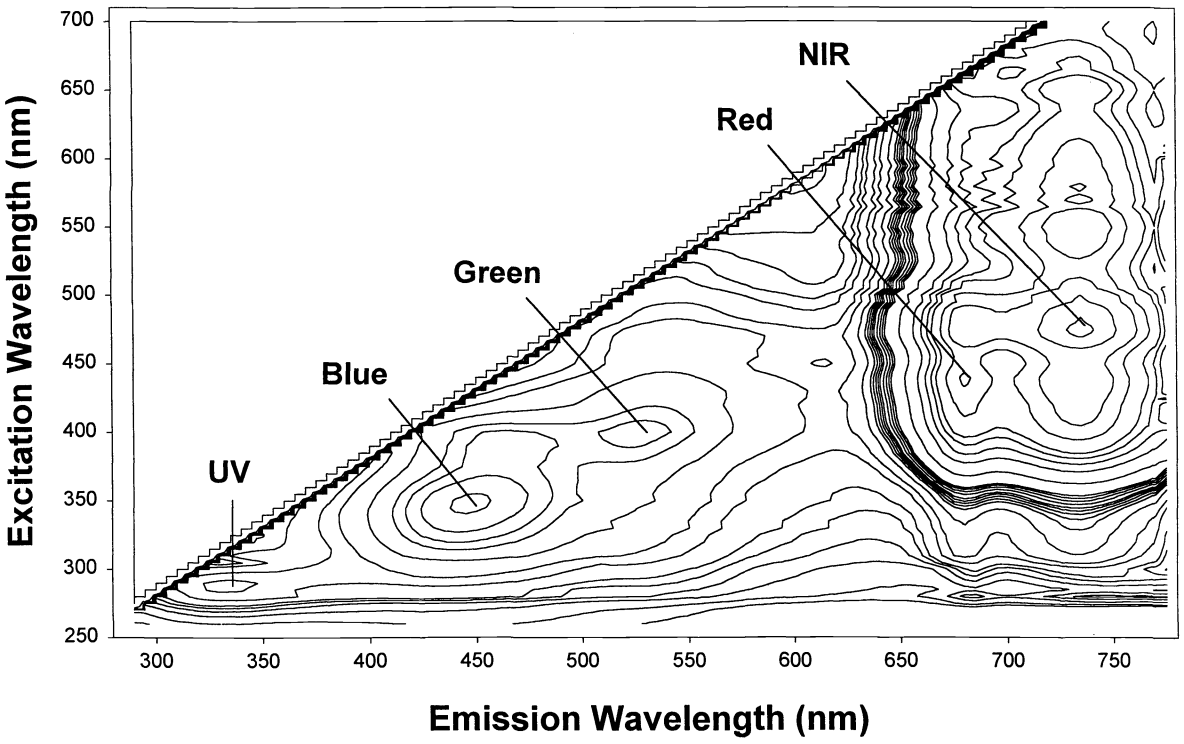
3. RESULTS AND DISCUSSION

3.1 Fluorescence EEM

The fluorescence emission from vegetation varies depending on the wavelength of excitation. The fluorescence EEM (excitation and emission matrix) is a 3-d mesh plot with the x and y axises corresponding to excitation and emission wavelengths while the z-axis corresponds to intensity. The fluorescence EEM for a healthy soybean leaf is shown in fig. 1 along with the corresponding contour plot in fig. 2. *In vivo* soybean leaf fluorescence emissions were determined in the wavelength range from 300 to 800 nm (5 nm intervals). While excitation characteristics were determined in the wavelength range from 250 to 660 nm (5 nm intervals). From these kinds of plots we can predict how vegetation will respond to various laser excitation wavelengths. With current technology the EEM is time consuming to acquire and process, and as a result, is only performed as a means to select excitation wavelengths which have high potential to differentiate vegetation treated with

Fluorescence Excitation and Emission Matrix





various types of stress. The optimal excitation wavelengths to maximize emission intensities for each of the five emission bands are indicated in figure 2. Most frequently ratios of fluorescence bands are used to differentiate stressed vegetation resulting in a compromise of excitation wavelength to receive multiple fluorescent emissions. Our most recent studies utilized 380 nm as an excitation wavelength which increases the emission in the green, red, and NIR regions while decreasing the contribution in the blue by only 25%. Ideally, one needs to determine which bands of fluorescence are most affected by a plant stressor and select excitation wavelengths which maximize differences in these bands.

3.2. UV and Blue Bands of Fluorescence

Illumination of a healthy soybean leaf with 280 nm radiation produced multiple fluorescent emissions in the UV, blue, green, red, and NIR regions of the spectrum (fig. 3). The excitation spectrum shown in fig. 3 indicates dual excitation maxima, the first centered at 232 nm, and the second centered at 284 nm. The symmetry and location of these two excitation maxima closely resemble the absorption maxima of TRP (in aqueous solution) which is the primary chromophore within proteins for this fluorescence emission[10].

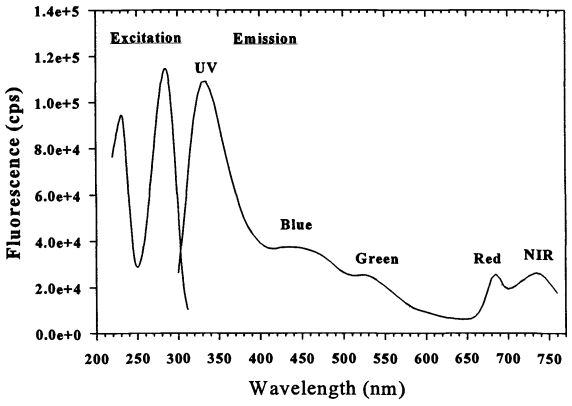


Figure 3. Healthy soybean leaf fluorescence characteristics. Excitation spectrum was obtained at a fixed emission wavelength of 335 nm. Emission spectrum was obtained at a fixed excitation wavelength of 280 nm.

Fluorescence emission spectra for soybeans treated with varying levels of nitrogen were recorded at an excitation of 280 nm (fig. 4) and at 340 nm (fig 5). Clear trends were apparent with the magnitude of fluorescence at 335 nm decreasing with nitrogen level.

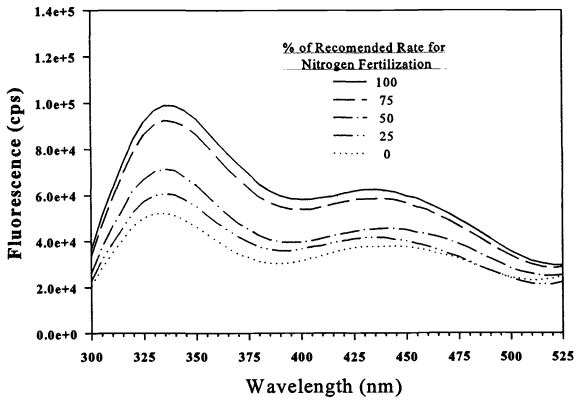


Figure 4. Fluorescence emission spectra (excitation at 280 nm) for soybeans treated with varying levels of nitrogen. Each curve represents the mean of eight replications.

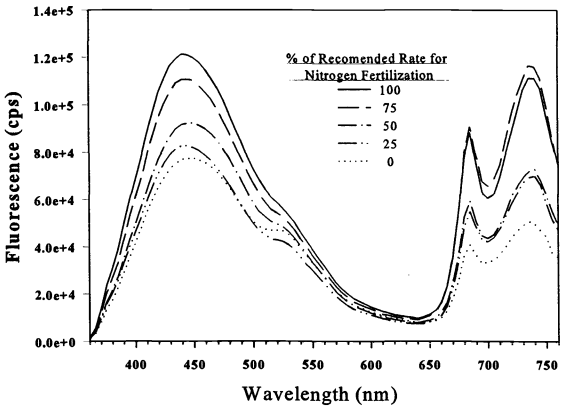


Figure 5. Fluorescence emission spectra (excitation at 340 nm) for soybeans treated with varying levels of nitrogen. Each curve represents the mean of eight replications.

The asymeteric double sigmoidal curve fit routine was used to deconvolute the individual fluorescence spectra. Ratioing the deconvoluted curve areas significantly improved the relationship between the fluorescence ratio (335 nm / 440 nm) and nitrogen fertilization level (table 1). This fluorescence ratio was more sensitive to nitrogen fertilization level than measurements of chlorophyll concentration or gas exchange. Gas exchange measurements provided significant separation of the high and low treatments, while correlation analysis did not indicated a strong relationship to the magnitude of fluorescence bands or ratios of these bands. The highest correlation between gas exchange and fluorescence occurred with the fluorescence ratio of 440/600 when excited at 340 nm (r = 0.60). These findings are consistent with those reported in previous studies[2] although this relationship was not as strong.

% Optimal Fertilization Rate	Chlorophyll a ($\mu g/cm^2$)	% Protein (based on dry leaf weight)	Fluorescence @ 335 (ex. 280 nm)	Fluorescence Ratio 335/440 (ex. 280 nm)	Fluorescence Ratio 440/600 (ex. 340 nm)	Photosynthesis (μMoles $CO_2 m^{-2} s^{-1}$)
100	4.427 a	28.85 a	98986 a	2.024 a	4.246 a	37.721 a
75	3.427 b	29.16 a	92495 a	1.666 b	4.229 a	30.426 b
50	2.911 b	24.64 b	71522 ab	1.348 bc	3.807 b	29.926 b
25	2.713 b	21.43 b	60699 b	1.130 cd	3.723 b	26.504 b
0	1.335 c	16.51 c	52187 b	0.906 d	3.037 c	19.730 c

Table 1 Student-Newman-Keuls multiple range test was performed at the 95 % confidence level. Means with the same letter are not significantly different.

The relationship between the *in vivo* fluorescence ratio of 335nm/440nm (ex. 280 nm) and the concentration of total leaf protein was explored (figure 6). Since 50 to 80% of leaf nitrogen is allocated to photosynthetic proteins including rubisco whose concentration increases with increasing nitrogen supply[22], fluorescence could be a more sensitive tool to assay the physiological productivity of the leaf than a single gas exchange measurement. As evidenced by these studies UV band and to a higher degree the ratio of UV band to the blue band fluorescence increases with increasing protein concentration. This relationship becomes more variable for higher protein concentrations for reasons yet to be determined. Further studies are required to develop the potential of this technique to encompass not only greenhouse but field grown vegetation.

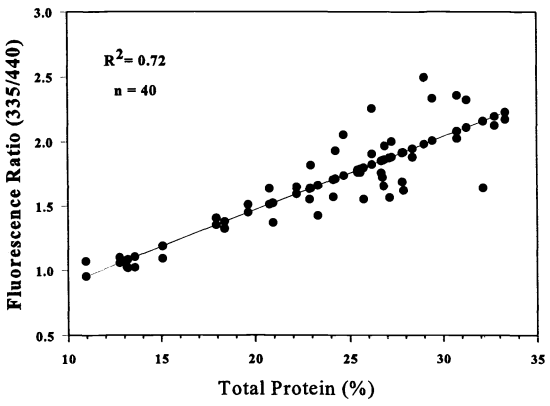


Figure 6. The relationship between total protein as a percent of dry leaf weight and the deconvoluted ratio of UV to blue bands of fluorescence.

3.3 Red and NIR Emission Bands

Illumination of vegetation from 250 nm to 675 nm produced multiple fluorescent emissions in the red (~685 nm) and NIR (~740 nm) regions of the spectrum. Excitation spectra for each of these fluorescence emissions are shown in fig. 7 and fig. 8 respectively. Each curve has multiple peaks which relate to the combined absorption of light by plant pigments and its subsequent use in energy transfer reactions of photosynthesis. The intensity of the fluorescence emission as a function of excitation wavelength was directly related to concentrations of plant pigments and inversely related to the efficiency of energy transfer in photosynthesis.

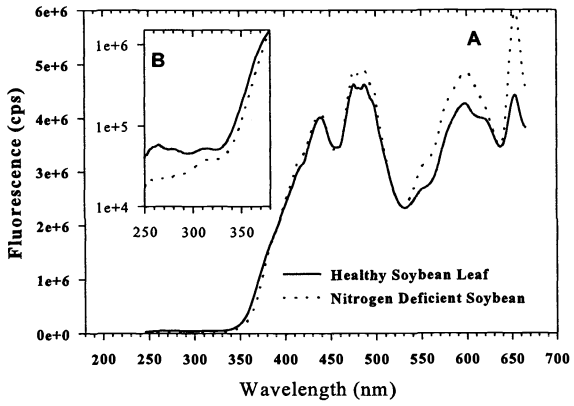


Figure 7. Healthy vs. nitrogen-deficient soybean leaf fluorescence characteristics. Excitation spectrum was obtained at a fixed emission wavelength of 685 nm (red band emission maximum). The inset in this figure was plotted on a log scale to better display treatment differences.

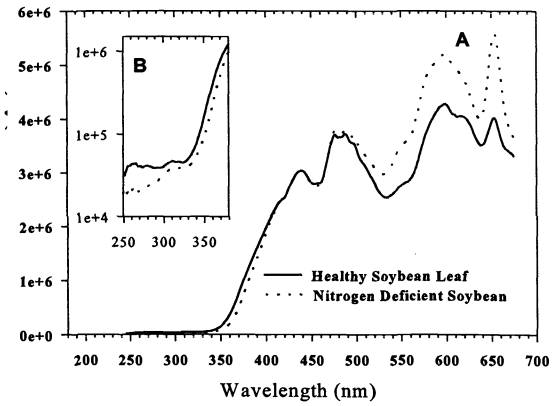


Figure 8. Healthy vs. nitrogen-deficient soybean leaf fluorescence characteristics. Excitation spectrum was obtained at a fixed emission wavelength of 740 nm (NIR band emission maximum). The inset in this figure was plotted on a log scale to better display treatment differences.

When assessing fluorescence information in the red and NIR bands independently, the greatest differences due to nitrogen fertilization level occur at excitation wavelengths ranging from 560 to 670 nm. The most significant differences occur when these bands are excited at or near 600 nm and 660 nm. Information in the red and NIR bands of fluorescence are influenced by short term fluctuations in the rate of photosynthesis and as a result are highly variable. Whereas information received in the UV, blue, and green bands of fluorescence relate to compounds whose concentration tend to vary over a much longer time frame. As a result, these emissions are much more stable.

3.4. Ratio Spectra

Relative differences between these excitation spectra are better seen through the ratio spectra where the red excitation spectrum was divided by the NIR excitation spectrum (fig. 9). The distribution of peaks and valleys in this ratio spectrum yield striking similarities to the action spectrum of photosystem I and II (data taken from [17]) (fig. 10). Peaks in the ratio spectrum correspond to absorption maxima of plant pigments that make up the photosynthetic apparatus. It is possible that some of the variation in the ratio spectrum is due to the concentrations of pigments within each of these two photosystems. From the ratio spectra it is apparent that relative differences between the fluorescence emission intensity at 685 nm to that of 740 nm are not constant and depend on the wavelength of excitation.

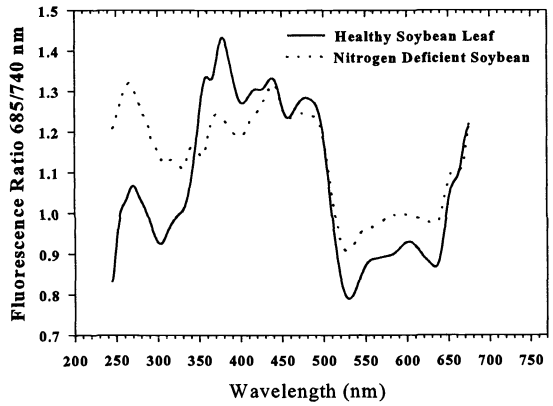


Figure 9. Healthy vs. nitrogen-deficient soybean leaf fluorescence characteristics. Ratio spectrum was obtained by dividing the 685 nm excitation spectrum by the corresponding 740 nm excitation spectrum.

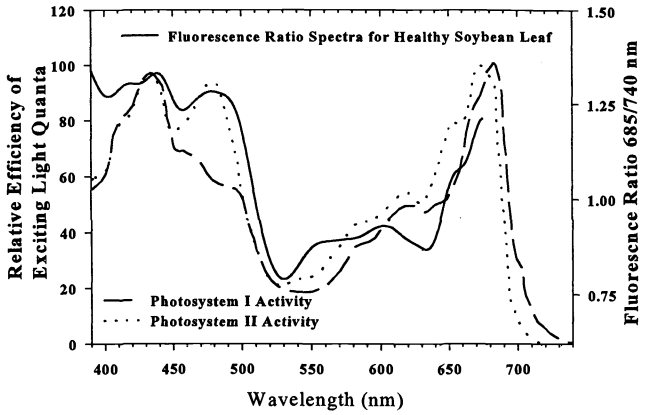


Figure 10. Comparison between the fluorescence ratio spectrum of a healthy soybean leaf and the action spectra for each of the two photosystems (data taken from [17]).

Differences are noted in the ratio spectra between the control and nitrogen-deficient soybean leaf in the excitation wavelength region from 250 nm to 300 nm. However, emission intensities are extremely low in this area and may not be detectible at the canopy level. Excitation areas to avoid include regions where the two curves in fig. 9 converge. From these data it would appear that an excitation area at or near 380 nm would yield substantial differences in the red to NIR fluorescence emission ratio in addition to providing information in the blue and green fluorescent regions.

4. CONCLUSIONS

The UV band fluorescence emission *in vivo* adequately separates soybean vegetation grown at varying levels of nitrogen. This fluorescence emission from intact vegetation decreases with a decreased supply of nitrogen. The relationship between fluorescence and nitrogen fertilization level was improved by deconvolution and the subsequent ratio of UV to blue fluorescence maxima. Since a large portion of the nitrogen in plants is present in the form of amino acids existing free or combined to form proteins, it follows that total protein concentrations decrease with decreasing availability of nitrogen.

Portable ground based instrumentation could easily be fabricated and could prove useful in assessing plant vigor. A rapid quantitative measure of nitrogen status could prove useful to many farming systems where substantial investments are made in the application of nitrate fertilizers. As far as legume crops are concerned, N_2 from the atmosphere can be made available to the plant if the roots are adequately inoculated with nodules from nitrogen fixing rizobium. A rapid procedure for assessing leaf nitrogen would be useful in determining problem spots in the field where incomplete inoculation can be supplimneted with organic or chemical fertilizers to improve soil health and crop yield. Economic and environmental benefits would result in variable management and application of both chemical and organic fertilizers.

The data presented here strongly supports our initial belief that physiological changes in plants as the result of perturbations in environmental conditions are manifested by changes in fluorescence spectra especially if the environmental changes affect photosynthesis. The differences among plant types in terms of photosynthetic efficiency and pigment composition result in unique fluorescence spectra. The relationships between photosynthesis, fluorescence, and plant vigor, allow studies to be made with fluorescence measurements which will permit an improved understanding of some of the fundamental mechanisms underlying the changes occurring in plants as the result of environmental fluctuations.

5. REFERENCES

1. Chappelle, E., F. Wood, J. McMurtrey, and W. Newcomb, "Laser induced fluorescence (LIF) of green plants. II: A technique for the remote detection of plant stress and species differentiation," Appl. Optics, 23: 134-138, 1984.

2. Chappelle, E., J. McMurtrey III, and M. Kim, "Identification of the pigment responsible for the blue fluorescence band in the laser induced fluorescence (LIF) spectra of green plants, and the potential use of this band in remotely estimating rates of photosynthesis," Remote Sens. Environ., 36: 213-218, 1991.

3. Chappelle, E.W.,Corp, L.A., Kim, M.S., and McMurtrey, J.E., "Significance of the blue band in the laser induced fluorescence (LIF) spectra of green vegetation," *Proc. Internat. Geoscience and Remote Sens. Symp.* 1333-1336, 1993.

4. Lang, M., Stober, F., Lichtenthaler, H.K., "Fluorescence emission spectra of plant leaves and plant constituents," Radiat. Environ. Biophys., 30,333-347, 1991.

5. Lichtenthaler, H. K., Stober, F., Buschmann, C., Rinderle, U., Hak, R., "Laser-Induced chlorophyll fluorescence and blue fluorescence of plants," *Proc. Internat. Geoscience and Remote Sens. Symp.* 1333-1336, 1990.

6. Cerovic, Z., M. Bergher, Y. Goulas, S. Tosti, and I. Moya, "Simultaneous measurement of changes in red and blue fluorescence in illuminated isolated chloroplasts and leaf pieces: The contribution of NADPH to the blue fluorescence signal," Photosynthesis Research, 36: 193-204, 1993.

7. Cerovic, Z.G., Morales, F., Moya, I., "Time-resolved spectral studies of blue-green fluorescence of leaves, mesophyll and chloroplast of sugar beet (Beta vulgaris L.)," Biochimica et Biophysica Acta., 1188:58-68, 1994.

8. Morales, F., Cerovic, Z.G., Moya, I., "Characterization of blue-green fluorescence in the mesophyll of sugar beet leaves affected by iron deficiency," J. Plant Physiol., 106:127-133, 1994.

9. Lichtenthaler, H. K., M. Lang, M. Sowinska, F. Heisel, and J. A. Miehe, "Detection of vegetation stress via a new high resolution fluorescence imaging system," J. Plant Physiol., 148:599-612, 1996.

10. Barenboim, G.M., Domanskii, A.N., Turoverov, K.K, *Luminescence of Biopolymers and Cells*, Plenum Press, New York, Chap. 1, 1969.

11. Wetlaufer, D. B. , "Ultraviolet spectra of proteins and amino acids", Adv. Protein Chem., 17: 303-390, 1962.

12. Corp, L., E. Chappelle, J. McMurtrey, M. Kim, "A New Fluorescence Band Obtained by the Excitation of Plants at 280 nm and its Implications to the Remote Assessment of Vegetation", *Proc. Internat. Geoscience and Remote Sensing Symp.*, 2: 986-989, 1994.

13. Bose, S., "Chlorophyll Fluorescence in Green Plants and Energy Transfer Pathways in Photosynthesis", Photochem. Photobiol., 36: 725-731, 1982.

14. Lictenthaler, H., U. Rinderle, "Role of chlorophyll fluorescence in the detection of stress conditions of plants", CRC Critical Rev. Anal. Chem., 19: 29-85 1988.

15. McMurtrey, J.E., Chappelle, E.W., Kim, M.S., Mesinger, J., Corp, L.A., "Development of algorithms for detecting nitrogen fertilization levels in field corn (Zea mays L.) with laser induced fluorescence", Remote Sens. of Environ., 47: 36-44, 1994.

16. Heisel, F., Sowinska, M., Miehe, J.A., Lang, M., Lichtenthaler, H.K., "Detection of nutrient deficiencies of maize by laser induced fluorescence imaging", J. Plant Physiol., 148:622-631, 1996.

17. Ried, A., "*Proc. 2nd Int. Congr. Photosynthesis Res.*", F. Forti, M. Avron, and A. Melandri, edition, 763-772, 1972.

18. Corp, L.A., McMurtrey, J.E., Chappelle, E.W., Kim, M.S., Daughtry, C.S.T., "Optimizing fluorescence excitation wavelengths for the detection of stress in vegetation", *Proc. Internat. Geoscience and Remote Sens. Symp.* 3:1812-1815, 1996.

19. Lichtenthaler, H.K., "Chlorophylls and carotenoids: pigments of photosynthetic biomembranes", Methods Enzymol. 148:350-382, 1987.

20. Bellomonte, G., Constantini, A., Giammarioli, S. , "Comparison of modified automatic dumas method and the traditional Kjeldal method for nitrogen determination", J. Assoc. Off. Anal. Chem. 70:227-229, 1987.

21. Hikosaka, K., Terashima, I., "A model of the acclimation of photosynthesis in the leaves of C_3 plants to sun and shade with respect to nitrogen use", Plant Cell and Environ. 18:605-618, 1995.

22. Lawlor, D.W., Kontturi, M., Young, A.T., "Photosynthesis by flag leaves of wheat in relation to protein, ribulose bisphosphate carboxylase activity and nitrogen supply", J. Exp. Botn. 40:43-52, 1989.