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Steady-state natural fluorescence

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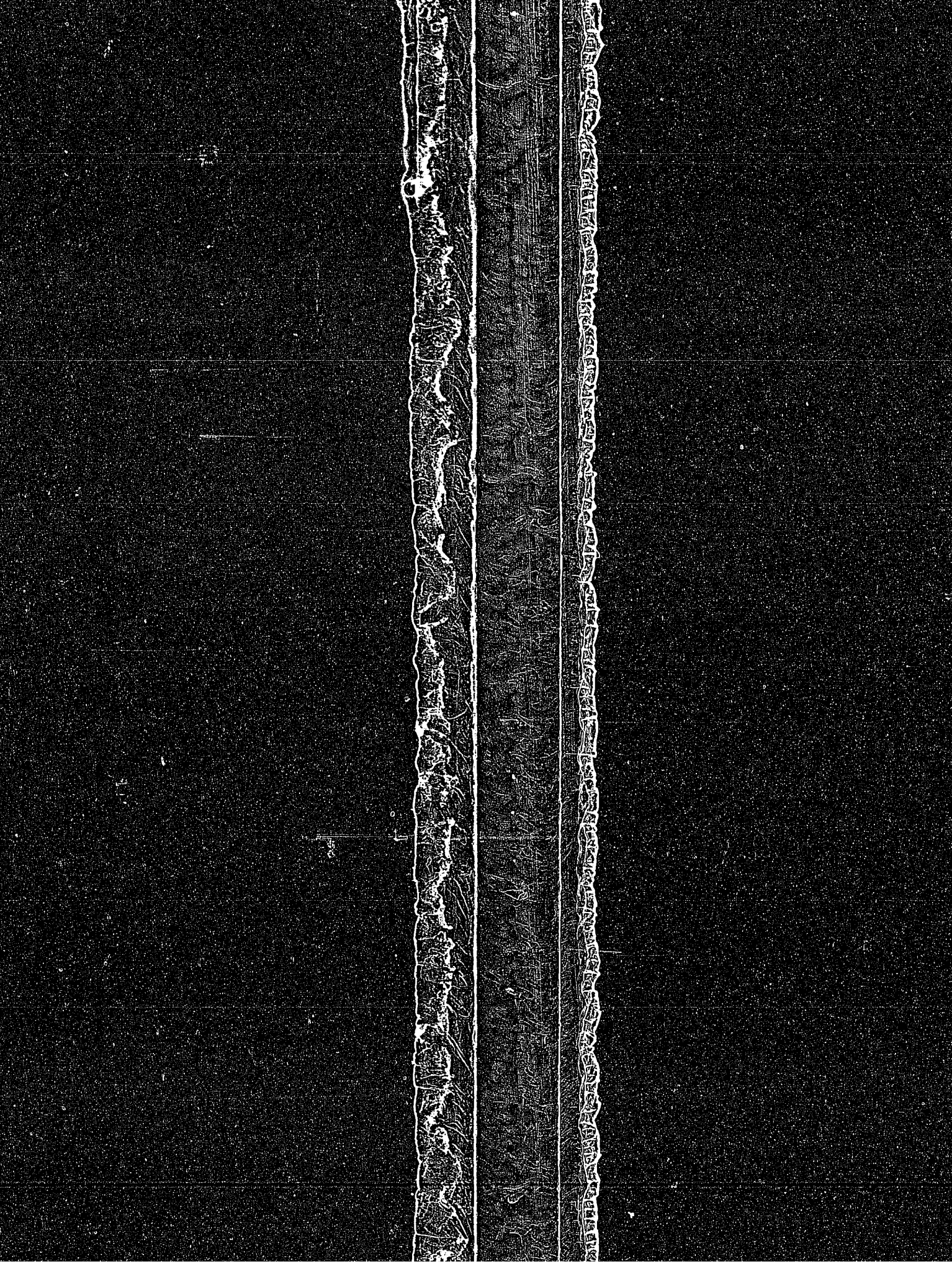

STEADY-STATE NATURAL FLUORESCENCE

BY

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DISSERTATION

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This dissertation has been examined and approved.

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ABSTRACT

STEADY-STATE NATURAL FLUORESCENCE

by

Arnold Francis Theisen

University of New Hampshire, December, 1994

This study employed a manifold analytical approach to the application of multiple excitation and emission wavelengths of steady-state natural fluorescence for the detection of stressed and damaged vegetation. This investigator has attempted to answer questions put forth in terms of the following Null Hypothesis: **Natural fluorescence emission, measured in the steady-state from living, attached or freshly excised needles of eastern white pine (*Pinus strobus* L.) or leaves of common beans (*Phaseolus vulgaris* L.), cannot be used in the form of complete spectra or in ratios of discrete wavelengths as indicators of stress from abiotic sources such as ozone or cadmium.** The Null Hypothosis was rejected in the case of white pine subjected to short-term ozone exposure. In experiments conducted in 1992 and 1993, mean red/far-red (R/FR) fluorescence ratios for pine measured 24 hours after fumigation were found to be significantly higher than the R/FR ratio means from pre-fumigation measurements. The fluorescence changes were most likely related to the effects ozone has on the water-splitting molecule and the thylakoid membrane rather than on a reduction in chlorophyll content. The Null Hypothesis was also rejected for the case of beans grown in cadmium doped soil. Strong correlations were found between R/FR ratio means and a series of cadmium treatments ranging from 0 to 50 mg Kg^{-1}. The R/FR ratio means were also strongly correlated with bean chlorophyll content indicating that the cadmium was producing previsual chlorophyll losses. In a second experiment, strong correlation between fluorescence maxima and cadmium treated beans that did not have losses of chlorophyll suggest that fluorescence intensity may be used to detect stressed conditions

prior to chlorophyll loss. A technique has been successfully demonstrated for detecting differences in pigment absorption *in vivo* in short- and long-term water stressed beans using the 3rd derivative of excitation spectra from excitation-emission-matrix data sets.

INTRODUCTION

Plants in general, and of more specific importance to humanity, those plants that comprise agriculture and silviculture are in normal dynamic balance with natural stressing factors such as floods, droughts, insects, and disease. The first two factors have been caused by local climatic changes since before recorded history. While local weather pattern changes are recognized to be related to, or be a result of, global climatic changes, in recent years it is claimed that these changes are being influenced by more than natural events. There is also an increase in vegetation subjected to direct contact with anthropogenic agents. The most publicized of these anthropogenic agents are the result of the burning of fossil fuels for industry and transportation. With the end of the Cold War, the extent of the lesser known contamination with heavy metals and organic solvents, in localized areas used for military bases, weapons research and production, and non-military industrial processes, is an important subject of study.

When the symptoms of stress due to natural or other causes are expressed visibly, it is often too late to save the affected plants or to separate the primary cause from contributing causes of the resulting damage. Weakened plants are less able to sustain a defence against attack by insects and disease. The visible expressions of these secondary stressing factors further confuses possible diagnosis. Significant losses of this part of the biosphere are important not only because of the economic value of timber, fiber, and food crops for human consumption, but also because they represent losses of food and shelter for all other fauna our planet is capable of supporting. In a more fundamental way, vegetation plays a significant role in global carbon cycling and in the process provides the oxygen without which animal life cannot exist.

Because timely remedial action can reduce losses, methods are needed to monitor the physiological status of vegetation to determine the presence or extent of stressing conditions without contributing to or exacerbating those conditions. The loss of

vegetation in and of itself is not the only importance to the environment. If vegetation losses or even reduced production in areas suspected to be contaminated by anthropogenic agents can be used as an indicator of the scope of the contamination, efforts to remove or neutralize the contamination can be more efficient and cost effective. The method or methods to be developed should be capable of following the growth cycle of a single plant non-destructively, and further, be capable of an expanded scope to include assessment at the canopy, forest or field, and, ultimately, global level.

Purpose of this Study

This study addresses the value of employing steady-state natural fluorescence measurements for the assessment of the physiological status of vegetation from two specific abiotic stressing factors. To evaluate the effects of anthropogenic agents related to the burning of fossil fuels, eastern white pine (*Pinus strobus* L.) was subjected to short-term elevated levels of ozone, and the results of this evaluation are reported in Chapter I. Chapter II is an assessment of common beans (*Phaseolus vulgaris* L.) grown in soil doped with a heavy metal (cadmium). This investigator has attempted to answer questions put forth in terms of the following null hypothesis:

> Natural fluorescence emission, measured in the steady-state from living, attached or freshly excised needles of eastern white pine or leaves of common beans, cannot be used in the form of complete spectra or in ratios of discrete wavelengths as indicators of stress from abiotic sources such as ozone or cadmium.

Fluorescence and Chlorophyll Fluorescence

Fluorescence arises from the excitation of an electron in an atom, ion , or molecule, from the ground state (S_0) to some level of an excited singlet state (usually S_1 or S_2) by the energy it receives from a photon, and the subsequent path back to the ground state, first by internal conversion to heat, until the excited electron reaches the lowest level of S_1. From that energy level it makes the photon-releasing radiative transfer back to S_0 (figure 1). The released photon has a longer wavelength than the exciting photon resulting from the loss of energy as heat. The difference in wavelength is known as a Stokes' shift (Hercules, 1966; Lumb, 1978). The loss of energy by heat takes place within 10^{-12} seconds and the radiative decay, or fluorescence, occurs within 10^{-9} seconds. An alternative way of expressing this phenomenon is that a singlet-singlet transition produces "prompt" fluorescence.

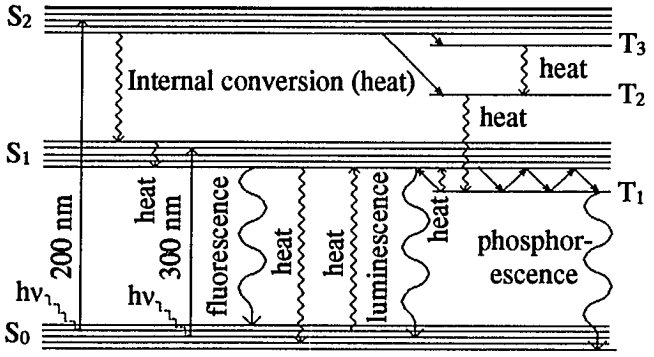

Figure 1. Jablonski Diagram: The electron energy action and emission upon photon excitation. S_0 is the ground state, S_1 is the first singlet excited state, S_2 is the second excited state, and $T_{1\text{-}3}$ are the excited triplet states. The figure combines elements from similar diagrams in Hercules (1966) and Lumb (1978).

When an excited electron is passed from a singlet excited state (i.e. S_2) to a triplet excited state (T_3 or T_2), decays through heat loss to the lowest triplet level (T_1), and returns from that triplet state, radiatively, to the ground state (S_0), the emission is termed phosphorescence. The decay time is much longer than for fluorescence and can take from milliseconds to tens of seconds. While a singlet-triplet transition is formally forbidden by quantum mechanics, Lumb (1978) explains that there is still a finite rate constant of

approximately 10^6 s^{-1}, and the long-lived phosphorescence emission is the result of this large rate constant. The phosphorescence transitions also produce an emission spectrum different from fluorescence.

Luminescence has had multiple meanings applied to it. D.M. Hercules (1966) states that luminescence is the radiation emitted by a molecule or atom after it has absorbed energy to go to an excited state, and further that, as far as the analytical chemist is concerned, luminescence is the inclusive term for fluorescence and phosphorescence. M.D. Lumb (1978) subdivides luminescence into photo-, cathodo-, radio-, bio-, thermo-, sono-, and electroluminescence according to the exciting process. When the intention is to relate the spectral emission studies of plants to the natural environment, it is assumed that the excitation energy will be from UV or visible light, which would therefore refer to photoluminescence. Photoluminescence is further subdivided by Lumb dependent on whether the material being excited is organic or inorganic. That inorganic materials are held together by ionic and covalent bonds, and organic materials are held together by van der Waals forces between molecules is the basis for Lumb creating these separate categories. Whereas the emissions of inorganic materials are associated with defects or impurities in a crystal lattice, or the excited states of isolated atoms or ions, organic materials owe their emissions to the excited states of delocalized π-electrons of whole molecules. In the study of vegetation, luminescence refers to the delayed fluorescence phenomenon (figure 1).

Energy from a wide range of the UV and visible spectrum can be absorbed for use by leaves, needles, or even bark by chlorophyll and other molecules. This is called light harvesting, and the molecules responsible are known collectively as light harvesting complexes (LHCs) and antenna pigments. The absorbed energy is then transferred from one molecule to another, sometimes in a so-called "random walk" (figure 2), until it reaches a special chlorophyll molecule known as a reaction center (RC). This "bucket brigade" passage of energy is called the Förster mechanism after its discoverer (Junge,

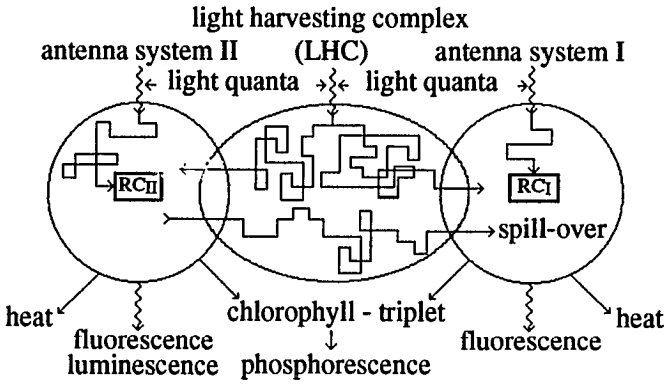

Figure 2. Tripartite Model for the Antenna System: Absorbed quanta are funneled by multiple resonant energy transfer steps into reaction centers (RC_{II} and RC_{I}). Energy is lost by the processes of heat, fluorescence, luminescence, and phosphorescence (Junge, 1977, figure 2).

1977). When the RC receives energy to excite one of its π-electrons the excitation energy is not passed on. Rather, an electron is lost to a pheophytin molecule oxidizing the RC. The oxidized RC cannot accept additional excitation energy from the LHC and antenna pigments and, if the RC is not returned to the reduced state rapidly enough, the excited π-electron of this chlorophyll molecule can drop to the ground state releasing a new photon known as chlorophyll fluorescence.

In a healthy, mature, green leaf in direct sunlight the apparatus of photosynthesis should be functioning to maximum capacity, assuming that there is no lack of water or required nutrients, that the leaf is adapted to function in direct sunlight and will therefore not be suffering from photoinhibition, and further that temperature and vapor-pressure-deficits are within optimal ranges. This leaf will absorb between 44 and 88 percent of the photosynthetically active radiation (PAR, 400-700 nm) available at its surface. Far-infrared radiation also reaches the leaf from the surrounding environment and is a contributing factor in raising electrons to excited states, higher levels within excited states, or even to different states (i.e. the triplet-singlet transition producing delayed fluorescence or luminescence; see figure 1). The leaf also emits far-infrared radiation at levels that, normally, exceed 50 percent of the total solar and far-infrared energy it receives, and can exceed 80 percent of the total received energy.

At moderately high light levels (800 mol quanta m^{-2} s^{-1}) only 10-50 percent of incident light energy is used for photosynthesis, and in full sunlight (2000 mol quanta m^{-2} s^{-1} the percentage is even lower. The excess energy is mainly lost as heat, with some fluorescence (Walker, 1992). Under these conditions the system is overexcited, all the RCs are oxidized most of the time, and excess excitation energy not lost as heat cannot be passed on as an electron to pheophytin, or is passed back to the LHCs or antenna pigments. The chlorophyll molecules will dissipate the excess energy as fluorescence rather than as phosphorescence or luminescence.

The Red/Far-Red Fluorescence Ratio

The ratio of red (ca 680 nm) to far-red (ca 740 nm) emission was recognized by Lichtenthaler et al. (1986) to be an indicator of physiological activity, in addition to being a measure of relative chlorophyll content. Incubating a leaf with the herbicide diuron (DCMU) greatly increases red and far-red emission intensity, although red emission is increased to a greater degree than is far-red emission (Lichtenthaler et al., 1986). Diuron acts to block the Q_B binding site, a major link in the electron transport pathway between photosystem II (PSII) and photosystem I (PSI), interfering with the electron transport process.

Fluorescence at 690 nm was compared to that at 735 nm by Lichtenthaler and Rinderle (1988) for several plants and different chlorophyll contents of those plants. For Norway spruce (*Picea abies* L. Karst.), the ratio F690/F735 varied directly with chlorophyll content which increases up to a point as a plant matures, and decreases under conditions of stress, such as mineral or water shortage or excess, as well as high light or heat. The increase in the F690/F735 ratio with loss of chlorophyll is explained by a decrease in self absorption by chlorophyll in the red spectral region (Krause and Weis, 1991). It is generally accepted that the fluorescence peak in the red portion of the

spectrum near 685 nm is principally due to PSII emission, and the fluorescence peak near 735 nm is principally due to PSI (Strasser and Butler, 1977b; Mullet et al., 1980; Stahl et al., 1988; Gruszecki et al., 1991), although there is still some disagreement as to whether PSI makes a significant contribution to the 735 nm emission peak at normal physiological temperatures (Krause and Weis, 1991). At normal physiological temperatures, 735 nm emission is less variable than 690 nm emission and the effectiveness of the F690/F735 fluorescence ratio, in relation to electron flow, may be due to low sensitivity of PSI fluorescence in response to PSI photochemistry changes, whereas PSII fluorescence is more responsive to photochemical change (Bradbury and Baker, 1981).

Fluorescence Measurements

The fluorescence measurements for the several studies reported here are made with a Perkin-Elmer MPF-44B[1] laboratory fluorescence spectrophotometer that is briefly described in each of the MATERIALS AND METHODS sections of the following Chapters. The data was collected either as individual emission spectra at specific excitation wavelengths or as excitation-emission-matrices (EEMs) where the emission data is collected over a specified range of wavelengths and the excitation is stepped through a range of wavelengths. A method of extracting more than spectral or ratio data from the EEM fluorescence data sets is discussed in Chapter III. Data collection with the spectrophotometer was augmented with an Apple IIe microcomputer and a Cyborg/ISAAC hardware/software laboratory interface. Software was developed and written by this author specifically for the tasks required. Further information about the interface of the spectrophotometer to the microcomputer is covered in Chapter IV.

1/ The use of trade names is for convenience and does not imply endorsement by the author or the University of New Hampshire.

Quality Assurance and Control

For an instrument, such as the laboratory fluorescence spectrometer, that produces output of the spectral properties of measured samples, it is paramount that wavelength control be accurate. Calibration procedures to test and set wavelength accuracy were conducted on this instrument when it was delivered to the U.S. Geological Survey several years ago and repeated periodically to retest that accuracy. No corrections have been required even though the instrument has been moved from Flagstaff, Arizona to Denver, Colorado, from Denver to the University of New Hampshire (UNH), and from UNH to Acadia National Park, Maine and back several times. Wavelength checks after each move confirm the stability of this aspect of the instrument

The absolute intensity of a standard is another matter, as this depends on many variables and adjustments; among them the quality of the excitation source (a Xenon lamp has a lifetime of approximately 1000 hours), the focus of the light from that lamp onto the sample, the quality of the detectors, the positioning of the detectors, amplifier adjustments, and slit widths. Slit widths are standardized for calibration and data collection to match the sampling interval (e.g. 5 nm slit widths for 5 nm measurement resolution). Amplifier adjustments include those made internally for the rarely required electronic alignment, and those made from the front panel to accommodate individual circumstances. Detectors are checked and adjusted if necessary for peak response after any major move. The focus of the light source is also checked and adjusted at the same times and periodically with use as changes can occur as the lamp ages. Once all the above items are brought to their optimum, a standard dye (rhodamine B) suspended in a non-fluorescent transparent plastic (polymethyl methacrylate) is measured for its intensity and wavelength response. Periodic remeasurement of this standard provides a quick check for proper adjustment.

CHAPTER I[2]

DETECTION OF CHANGES IN STEADY-STATE CHLOROPHYLL FLUORESCENCE IN *Pinus strobus* FOLLOWING SHORT-TERM OZONE EXPOSURE

SUMMARY

Steady-state chlorophyll fluorescence measured with a laboratory fluorescence spectrophotometer has been used successfully, in repetitive experiments, to detect changes in the red/far-red (R/FR) emission intensity ratio of white pine (*Pinus strobus* L.) induced by fumigation with ozone. Experiments were conducted in 1992 and 1993 as part of a study of the effects of short-term ozone exposure to native plants at Acadia National Park, Maine. In July, 1992, fluorescence measurements were made of clonal grafts (ramets) of native white pine, prior to, one hour after, and 24 hours after exposure to ozone (duration of 3 hours at 180 mm^3m^{-3}). The R/FR fluorescence ratio mean from the spectra collected 24 hours after exposure was significantly different from the pre-fumigation R/FR ratio mean ($P = 0.10$). In August, 1992, the R/FR ratio mean at 24 hrs post-fumigation was significant at $P = 0.05$. In August, 1993 measurements were made, prior to, one hour after, 24 hours after, and 48 hours after exposure, with a different set of white pine ramets exposed to 120 and 220 mm^3m^{-3} ozone for 3 hours. The R/FR ratio means at 24 hrs post-fumigation that were significant at $P = 0.05$ for the 120 mm^3m^{-3} ozone exposure and at $P = 0.20$ for the 220 mm^3m^{-3} ozone exposure. Differences between the R/FR ratio means of pre- and 24 hrs post-fumigation measurements for 120, 180, and 220 mm^3m^{-3} ozone indicate dose dependent responses.

2/ This Chapter is published in the *Journal of Plant Physiology* vol. 144(3).

The 220 mm^3m^{-3} ozone exposed ramets produced an R/FR ratio mean at 48 hrs post-fumigation that was lower than the 24 hrs post-fumigation R/FR ratio mean (0.50 › P › 0.25), suggesting a potential recovery of photosynthetic processes from the effects of ozone exposure. Red/far-red fluorescence ratios, derived from chlorophyll emissions at wavelengths corresponding to known atmospheric and solar absorption features, indicates that stress-induced fluorescence changes may be detectable in direct sunlight.

INTRODUCTION

Elevated levels of tropospheric ozone can have a direct stressing effect on plants (see the definition of stress in Lichtenthaler and Rinderle, 1988). Recognizing these effects in a previsual, yet non-destructive, fashion requires the use of specialized tools. One such tool, evaluated in this report, is the use of the red/far-red (R/FR) ratio of natural, steady-state chlorophyll fluorescence. R/FR ratios can be derived from emission spectral data collected from living, potted seedlings with a laboratory fluorescence spectrophotometer. The Null Hypothesis to be tested: Comparison of R/FR fluorescence ratios derived from data obtained prior to and following exposure of plants to predetermined levels of ozone will yield differences indistinguishable from any differences due to random causes.

Acadia National Park, Maine, one of the most visited parks in the United States, has the geographical disadvantage of being downwind of several heavily populated areas. Pollution generated in these areas moves up the coast and, as a result of photochemical processes, may produce elevated levels of ozone in the park during the summer growing season. Previous studies have potentially linked ozone to park vegetation problems (Treshow, 1986; Bennett et al., 1986; Sanchini, 1988). Therefore, in 1989 the National Park Service sponsored research at the University of New Hampshire on the effects of chronic (long-term) ozone exposure on native plant species occurring at Acadia National

Park. This study employed open-top chambers (Heagle et al., 1973; Heagle et al., 1979) in which selected native plants were subjected to controlled levels of ozone, both above and below measured ambient levels, over an entire growing season. The chambers were installed and maintained at Acadia National Park by researchers from the Boyce Thompson Institute for Plant Research, Ithaca, New York.

Fluorescence data, gathered for a number species at monthly intervals during the growing seasons of 1990 and 1991, indicated that a variety of stress factors (i.e. insect damage, chamber effects) can have as great an effect on R/FR ratio levels as the factor under direct control (i.e. ozone level). Analysis of the data provided results (not shown) indicating that multiple stress factors, including ozone exposure, produced appreciable fluorescence response in some species but not all. It was determined that in order to develop a better understanding of the direct impact of ozone on sensitive species, a much closer linkage must be made between the exposure to ozone at predetermined levels and the measurement of the fluorescence response. A short-term exposure experiment with immediate fluorescence measurements for selected species was designed to accomplish this goal.

Fluorescence emission spectroscopy, in which the excitation wavelength corresponds to a specific absorption maximum of the pigment of interest, has been used for several years for the study of plant function. H.I. Virgin (1956) found fluorescence a useful property of chlorophyll pigments that could be used to investigate physical and physiological characteristics in plant systems. The ratio of red (ca 680 nm) to far-red (ca 740 nm) emission was recognized by Lichtenthaler et al. (1986) to be an indicator of physiological activity, in addition to being a measure of relative chlorophyll content. By incubating a leaf with the herbicide diuron (DCMU), not only is the red emission intensity greatly increased, but fluorescence spectroscopy reveals that red emission is increased to a greater degree than is far-red emission (Lichtenthaler et al., 1986). Diuron acts to block the Q_B binding site, a major link in the electron transport pathway between

PSII and PSI (Lawlor, 1990), interfering with the electron transport process.

Lichtenthaler and Rinderle (1988) compared the fluorescence at 690 nm to that at 735 nm for several plants and different chlorophyll contents of those plants. For Norway spruce (*Picea abies* (L.) Karst.), the ratio F690/F735 varied directly with chlorophyll content which increases up to a point as a plant matures, and decreases under conditions of stress, such as mineral or water shortage or excess, as well as high light or heat. The increase in the F690/F735 ratio with loss of chlorophyll is explained by a decrease in self absorption by chlorophyll in the red spectral region (Krause and Weis, 1991).

It is generally accepted that the fluorescence peak in the red portion of the spectrum near 685 nm is principally due to PSII emission, and the fluorescence peak near 735 nm is principally due to PSI (Strasser and Butler, 1977b; Mullet et al., 1980; Stahl et al., 1988; Gruszecki et al., 1991). There is still some disagreement as to whether PSI makes a significant contribution to the 735 nm emission peak at normal physiological temperatures (Krause and Weis, 1991). At normal physiological temperatures, 735 nm emission is less variable than 690 nm emission and the effectiveness of the F690/F735 fluorescence ratio, in relation to electron flow, may be due to low sensitivity of PSI fluorescence in response to PSI photochemistry changes, whereas PSII fluorescence is more responsive to photochemical change (Bradbury and Baker, 1981).

In addition to interfering with electron transport, which will produce an increase in the F690/F735 ratio quickly, PSII herbicides, described by van Rensen (1989), induce in growing leaves, the formation of shade-type chloroplasts (Lichtenthaler and Rinderle, 1988). This response will also increase the F690/F735 ratio as shown in different spectra produced by sun and shade leaves (Lichtenthaler and Rinderle, 1988) with shade leaves producing slightly higher R/FR ratios than sun leaves. Upper and lower sides of leaves also produce differing spectra, with the lower sides producing much higher ratios than upper sides (Lichtenthaler et al., 1986; Lichtenthaler and Rinderle, 1988). Since the illumination conditions for sun and shade leaves, and upper and lower sides differ, leaves

adjust the chlorophyll content and chloroplast composition to make the best use of the quantity and quality of the light reaching the cells nearest those surfaces.

One of the advantages of the R/FR ratio method is that it is not confined to laboratory measurements. The emission in the red and far-red can be excited with monochromator- or filter-limited lamps, continuous or pulsed lasers, or direct sunlight. Lichtenthaler and Rinderle (1988) report the adaptation of this method to pulse amplitude modulation (PAM) techniques, and Zimmermann and Günther (1986) reported the successful use of the ratio method with a remote sensing laser instrument (LIDAR). The Fraunhofer line discriminator (FLD), an airborne remote sensing instrument, uses the passive Fraunhofer line depth method (Plascyk, 1975) for measuring steady-state fluorescence in full sunlight (McFarlane et al., 1980; Carter et al., 1990). The FLD uses the solar hydrogen α absorption feature "Fraunhofer line" at 656.3 nm (bandwidth at one-half maximum depth = 0.15 nm) on the blue shoulder of the red chlorophyll spectral emission to detect differing levels of chlorophyll fluorescence when the intensity within the Fraunhofer line is compared to the adjacent continuum (a measurement taken with a bandwidth of ca 1.0 nm centered on the Fraunhofer line). Another absorption feature that could be detected by such an instrument at ground level, or flown at low altitudes, is the atmospheric oxygen "line" in the far-red portion of the spectrum at 760 nm (bandwidth at one-half maximum depth = 1.4 nm, Kurucz et al., 1984). This line is close to the maximum emission of chlorophyll in the far red (ca 740 nm). The sensitivity of the prototype FLD to chlorophyll fluorescence emission changes at 656.3 nm and the greater bandwidth of the 760 nm feature lead to the conclusion that these Fraunhofer lines could be successfully exploited for the passive detection of R/FR fluorescence ratio levels for the monitoring of plant physiological status and/or relative chlorophyll levels.

MATERIALS AND METHODS

In 1992, grafted seedlings (ramets) of eastern white pine (*Pinus strobus* L.), derived from trees (ortets) in Acadia National Park were used in the short-term ozone exposure experiments. Pine ortets were classified in the field, when cuttings were collected, as "symptomatic" or "asymptomatic", according to the amount of foliar chlorosis and necrosis present. Although the relation between symptoms and causal agents was unclear, ozone was postulated to be involved. While recent work by Dr. William Merrill indicates that much of the observed symptoms are related to fungi and insects (personal communication, Bruce L. Nash), the present authors have chosen to retain the terms as a convenience.

Current-year needles of two-year old pine ramets were measured for their reflectance properties with a Visible/Infrared Intelligent Spectrometer (VIRIS) developed by the Geophysical Environmental Research Corporation; data on the net rates of photosynthesis were collected with a Li-Cor 6200 portable gas exchange analyzer; and fluorescence response was measured with a Perkin-Elmer MPF-44B Fluorescence Spectrophotometer using techniques described below (reference to brand names is made as a matter of convenience and does not imply endorsement by the University of New Hampshire). Randomly selected subsets of white pine used for the fluorescence measurements included 8 ramets from "symptomatic" ortets and 8 ramets from "asymptomatic" ortets. Controls were 8 ramets, 4 of each classification, that were to be left unfumigated. These three groups were further divided equally into two pretreatment subgroups that were kept in either ambient ozone level open-top chambers or in chambers supplied with air passed through charcoal filters (ca 50% ambient ozone levels) according to the environments in which they had been maintained throughout the growing season. The growth chambers in use at Acadia N.P., Maine were a type commonly employed for outdoor research where plants are grown in mostly natural conditions (Heagle et al., 1973; Heagle et al., 1979). Ozone was supplied from liquid oxygen using a Griffin

Model 1A generator, and levels in the chambers were measured with Thermo Environmental Instruments Inc, (TECO) Model 49 monitors. Pre-fumigation fluorescence measurements, one per ramet, were made from 0830 hours to 1200 hours after the plants had time to reach normal photosynthetic activity. Plants were then distributed to two chambers where they were fumigated from 1200 to 1500 hours. Fumigation was conducted in the two chambers with ozone levels of 180 mm^3m^{-3} maintained for 3 hours to simulate the average high concentration ozone episodes on record for Acadia National Park (personal communication, Robert Breen).

One hour post-fumigation measurements were made from 1600 to 1700 hours. Because of the low sun angle and shading at the site after 1700 hours it was decided that measurements of the control plants, corresponding to the post-fumigation measurements of the exposed plants, would not be valid as every effort was made to keep premeasurement conditions as consistent as possible even under the non-laboratory situation. The 24 hour post-fumigation measurements were begun approximately two hours early (1400 hours) to allow the measurement of all treated as well as all control plants. All measurement days in July and August of 1992 were sunny.

In August, 1993, the short-term experiment was repeated with a different set of white pine ramets from the park area that were not identified as either "symptomatic" or "asymptomatic". The trees were divided randomly into three groups of 6 ramets each, one group for control, and one group each for fumigation at 120 and 220 mm^3m^{-3} ozone for 3 hours. All ramets were kept in ambient conditions prior to the experiment. In addition to the pre-, 1 hr post-, and 24 hrs post-fumigation measurements, 48 hrs post-fumigation measurements were possible because of favorable weather conditions. Control plants were not measured at the 1 hour post-fumigation time in 1993 for the same reasons as were given for the 1992 measurements.

A light excluding extension was added to the sample compartment of the laboratory fluorescence spectrophotometer to accommodate whole potted plants. The

instrument has scanning monochromators for both excitation and emission, each with a measuring range from 200 to 900 nm. Both excitation and emission slit widths are adjustable for bandwidth limiting, and a ratio mode is used wherein the gain of the sample detector is controlled by a reference detector to minimize the effects of any potential drift in the excitation source lamp. The source lamp is a 150 watt Xenon powered by a current-stabilized supply. The signal detectors, sample and reference, are Hamamatsu R928 photomultipliers. For this study, emission intensity levels were collected at 5 nm increments, from 650 to 800 nm with an excitation wavelength of 470 nm. The emission spectra thus produced provided the data from which R/FR ratios were derived for analysis of PS II - PS I electron transport or total chlorophyll levels (Ruth and Weisel, 1993).

Even with all parameters of the instrument in alignment, the collected spectra may not be considered "true" representations of the emission and excitation spectra of the sample. The response characteristics of the instrument excitation source, photodetector, and excitation and emission optics, that vary with wavelength, should be taken into account so that resulting spectra, and derived ratios, may be confidently compared with like data collected with other fluorescence instruments. To correct the spectra for these variations, the response of rhodamine B dye, in high concentration such that it becomes a quantum counter, is compared to a constant output level over the excitation range to be used for data collection in order to provide an excitation correction file. An emission correction file is produced by comparing the light from a traceable standard tungsten lamp to the calculated blackbody emission of tungsten for the same color temperature. The raw spectral data are corrected using the values in the correction files before the spectra are stored on floppy disk. These correction files are updated with a Xenon lamp change or detector realignment.

Because of the small number of observations in each statistical sample of this study, the Student t test was used throughout. Use of the Student t test is dependent on

the assumption that each sample observation is derived by random selection from a normally distributed population. The R/FR fluorescence ratio data used for the analyses presented here meets the qualifying criteria described in Sokal and Rohlf (1981). The t value significance varies with the number of degrees of freedom of the statistical sample, and this was an important consideration for our study because sample sizes for 1992 and 1993 were different. Equations were obtained from Lapin (1983) and Sokal and Rohlf (1981), and Table G (Lapin, 1983) was the source of the Student's t distribution values.

To achieve an estimate of the potential usefulness of the passive Fraunhofer line depth method, R/FR ratios for emission spectra at an excitation wavelength of 470 nm were calculated from the intensities at 655 and 760 nm, corresponding to Fraunhofer lines centered near those wavelengths, and multiplied by 10 to adjust for the same order of magnitude, producing FLD-R/FR ratios.

RESULTS

In the 1992 experiment, fluorescence measurements were made on white pine ramets in July even though not all the needles were fully expanded. There were no statistically significant differences, in the R/FR ratio means, in comparisons between samples of different pretreatments at any stage of the experiment for either July or August ($P = 0.05$, data not shown). There were also no statistically significant differences between the R/FR ratio means of the "asymptomatic" and "symptomatic" classifications at any of the measurement times for either July or August ($P = 0.05$, data not shown).

The R/FR ratio means of the control ramets showed no change ($P = 0.80$, $df = 7$, $t = 0.11$) from the pre-fumigation measurement (0.24) to the 24 hrs post-fumigation measurement (0.23), while the R/FR ratio means for both "asymptomatic" and "symptomatic" ramets increased ($P = 0.20$, $df = 7$, $t = 1.57$ and $P = 0.25$, $df = 7$, $t = 1.20$,

respectively). Furthermore, since there was no statistical difference between the R/FR ratio means of the "symptomatic" and the "asymptomatic" ramets ($P = 0.80$, $df = 7$, $t = 0.14$), the R/FR ratio data for these samples were combined for the comparisons among the three measurement times, pre- (I), 1 hr post- (P), and 24 hrs post-fumigation (D). Plots of all 16 ramets of white pine are shown in figure 3a. The mean spectra for the pre- and 1 hr post-fumigation measurements appear essentially identical, while the mean spectrum for the 24 hrs post-fumigation measurement has a greater intensity in the red region near 685 nm. The statistical comparison of the R/FR ratio means (figure 3b) shows the same relationship, and further reveals that the 24 hrs post-fumigation R/FR ratio mean is significantly greater than those from the other two measurement times ($P = 0.10$, $df = 15$, $t = 1.75$).

In the August, 1992 experiment the needles of the white pine ramets were fully expanded and a second flush had begun on some of the clonal grafts. Plots of the mean spectra in figure 4a show a small increase in red emission from pre- to 1 hr post-fumigation, but a dramatic increase 24 hours after fumigation. There was, as in July, no statistical difference between the R/FR ratio means of the "symptomatic" and the "asymptomatic" ramets ($P = 0.50$, $df = 7$, $t = 0.67$), and therefore, all the ramets were combined for these comparisons. The increase in intensity in the red region at the 24 hrs post-fumigation measurement was greater in August ($P = 0.05$, $df = 15$, $t = 5.50$) than it was in July. There was a larger separation between the pre- and 1 hr post-fumigation mean spectra in August than in July, but neither of the differences between the R/FR ratio means for the two measurement times on the two dates were statistically significant. Before exposure, the R/FR ratio mean for the control ramets (0.20) was not statistically different ($P = 0.50$) from the R/FR ratio mean of the ramets that were fumigated (figure 4), and although the R/FR ratio mean of the control ramets increased slightly ($0.25 > P > 0.20$) at the 24 hour measurement to 0.26, it fell significantly ($P = 0.05$) below the R/FR ratio mean of the fumigated ramets.

A repeat of the short-term experiment in August, 1993 produced results in white pine comparable to those seen in 1992 at the 24 hrs post-fumigation measurement. The mean fluorescence emission spectra for the ramets exposed to 120 mm^3m^{-3} ozone for 3 hours are plotted in figure 5a, the R/FR ratio means are presented in a bar graph in figure 5b, and the mean fluorescence emission spectra and R/FR ratio means for the 220 mm^3m^{-3} ozone exposure are shown in figure 6. In contrast to the responses seen in the 1992 measurement results, the R/FR ratio means for both the 120 and 220 mm^3m^{-3} ozone exposures showed an apparently more immediate response to exposure, as the 1 hr post-fumigation R/FR ratio means were considerably greater than the pre-fumigation R/FR ratio means in both cases, producing t values of 2.14 ($0.10 > P > 0.05$, $df = 5$) and 1.40 ($0.25 > P > 0.20$, $df = 5$) respectively, versus 0.33 and 0.50 ($P = 0.50$, $df = 15$) for the July and August, 1992 180 mm^3m^{-3} ozone exposures. Comparison between the pre- and 24hrs post-fumigation R/FR ratio means for ramets fumigated at 120 mm^3m^{-3} ozone produced a result more significant ($t = 2.88$, $P = 0.05$, $df = 5$) than the July 1992 180 mm^3m^{-3} ozone fumigation ($t = 1.75$, $P = 0.10$, $df = 15$) or the August, 1993 220 mm^3m^{-3} ozone fumigation ($t = 1.67$, $P = 0.20$, $df = 5$), but less significant than the August, 1992 180 mm^3m^{-3} ozone fumigation ($t = 5.50$, $P = 0.05$, $df = 15$). The statistical data for the pre- and 24 hrs post-fumigation comparisons are compiled in Table 1.

The R/FR ratio mean for control ramets, measured in August, 1993, rose significantly ($0.10 > P > 0.05$) from the pre-fumigation level (0.08) to the 24 hrs post-fumigation level (0.13), but when a comparison is made between the spectra and R/FR ratio means for the 120 and 220 mm^3m^{-3} ozone treated ramets and the control ramets (figure 7), the control R/FR ratio mean can be seen to lie significantly below ($P = 0.05$) the other two R/FR ratio means.

The bar graphs of figure 8 show that, for the 120 mm^3m^{-3} ozone exposure, the 48 hour post-fumigation R/FR ratio mean was not statistically different from the 24 hour post-fumigation R/FR ratio mean ($P = 0.80$, $df = 5$, $t = 0.17$), but, for the 220 mm^3m^{-3}

ozone fumigated ramets, the 48 hour post-fumigation R/FR ratio mean was slightly lower than the 24 hour post-fumigation R/FR ratio mean ($P = 0.25$, $df = 5$, $t = 1.00$).

For the groups initially classified as "asymptomatic" and "symptomatic" and both pretreatment groups, none of the white pine ramets exposed to ozone in July or August, 1992 suffered foliar injury, and no foliar injury was observed in the 1993 treated pine even at the 48 hrs post-fumigation measurement. Gas exchange measurements for 1992 indicate that the rates of photosynthesis for all ramets were unaffected by ozone treatments (Eckert et al, 1993). White pine was not measured for gas exchange with the August, 1993 experiment. Preliminary assessment of concurrent spectral reflectance measurements indicate no obvious changes occurred in the visible portion of the spectrum in either 1992 or 1993 between the pre- and post-fumigation measurements.

DISCUSSION

The purpose of this study was to determine if pre-visual evidence of damage, related to ozone exposure, could be detected with steady-state fluorescence techniques, not to "prove" that ozone was causing damage to white pine. It is also unnecessary to show that white pine, allowing for genetic variability, is a species that can be highly sensitive to ozone (Chappelka and Chevone, 1992). Visible symptoms, such as chlorotic fleck, chlorotic mottle, and emergence tip burn, are accepted as diagnostic if biotic agents (mites and fungi can cause similar symptoms) are eliminated (Sinclair et al., 1987; Manion, 1991). Botkin et al. (1972) reported the appearance of visible symptoms (slight distal chlorosis) in the most sensitive foliage of their study of white pine the day following initiation of exposure to 750 mm^3m^{-3} ozone. Pell and Brennan (1973) and Schreiber et al. (1978), working with beans (*Phaseolus vulgaris* L. cv. Pure Gold Wax), noted the first visible symptoms of ozone damage between 21 and 24 hours after exposure. Wilkinson and Barnes (1973) report visible symptoms for pinto beans and

tobacco BEL-W3 in as little as one hour at 100 mm^3m^{-3}, but no visible symptoms for white pine exposed to 200 mm^3m^{-3} even after several hours. In another short-term ozone exposure experiment no visible macro- or microscopic damage to *Phaseolus vulgaris* (Saxa) beans was seen (Fischer, 1989), but deformed chloroplasts with stroma extensions and swollen intergranal thylakoids were found upon inspection with transmission electron microscopy (TEM). TEM techniques also revealed similar damage in rice (*Oryza sativa* L. cv. Koshihikari) exposed to ozone (Toyama et al., 1989) and spruce (*Picea abies* (L.) Karst) after ozone exposure (Schiffgens-Gruber and Luetz, 1992).

Ozone has been called one of the most toxic of atmospheric pollutants (Giamalva et al., 1985). It can even cause erosion and breakdown of leaf or needle cuticle contributing to visible symptoms with only external contact (Treshow and Anderson, 1991). Once ozone is inside the substomatal cavities of the mesophyll, it dissolves in and reacts with water to form several free radicals and ions which, in turn, oxidize membrane constituents such as sulfhydryl groups (Macdowall, 1965; Heath, 1975). This is, physiologically, the most severe form of damage to plants from ozone. According to Ledbetter et al. (1959), the membranes of chloroplasts are early targets of ozone-induced oxidation, with the thylakoid membranes especially sensitive. They suggest that this is probably due to oxidation of sulfhydryl enzymes, described by Slater (1949), embedded in thylakoid membranes. Mehlhorn et al. (1986) note that both proteins and lipids are also major oxidation targets of ozone.

The assumption that exposure of plants to ozone will produce a change in their natural steady-state chlorophyll fluorescence emissions, and therefore alter their R/FR ratios, is based on what is known or predicted about the physical and biochemical effects of ozone on plant tissue. Exposure of guard cells of stomata to ozone has been shown to decrease their turgidity causing them to close and limit further uptake of atmospheric gases, including ozone (Mansfield, 1973). The manner in which ozone reacts with stomatal cells and the resulting effect on fluorescence have been discussed by several

investigators. Maier-Maercker (1989) notes the partial delignification of the exterior walls of the stomatal apparatus; toxic derivatives of ozone were shown to cause changes in stomatal cell wall properties and stomatal closure (Moldau et al., 1990); and Aben et al. (1990) demonstrated that, in beans (*Vicia faba* L.), stomata were more sensitive to ozone exposure than was the photosynthetic system. Farage et al. (1991), working with wheat (*Triticum aestivum* L. cv. Avalon), noted a decrease in stomatal conductance associated with a decrease in with CO_2 uptake after fumigation with either 200 or 400 mm^3m^{-3} ozone for 4 to 16 hours. Lehnherr et al. (1987) also noted increased stomatal diffusive resistance (reduced stomatal conductance) in wheat, in ozone-enriched air, associated with an *in vivo* increase in the activation of rubisco with increasing ozone concentrations. McFarlane et al. (1980) demonstrated a high correlation between increases in red (656.3 nm) fluorescence emissions and increased diffusive resistance (the inverse of stomatal conductance) in drought stressed citrus.

At the macroscopic level, we observed no visible symptoms in the white pine one, 24, or 48 hours following fumigation in July or August, 1992, or August, 1993. Our data do show a significant increase in R/FR fluorescence ratio means at the 24 hour measurement in July ($P = 0.10$) and August of 1992 ($P = 0.05$) and for the 120 mm^3m^{-3} ozone exposure in August, 1993 ($P = 0.05$), although the 220 mm^3m^{-3} ozone exposure produced an increase that was not as great ($P = 0.20$). The lower response in July, 1992 is reasonable considering the less than fully expanded needles which would have exposed fewer stomata to the ozone. The reduced response at 220 mm^3m^{-3} ozone exposure compared to the 180 and 120 mm^3m^{-3} ozone exposures of August, 1992 and August, 1993, respectively, is consistent with the conclusions of Schreiber et al. (1978) that chronic exposure to lower ozone concentrations can be more injurious than brief exposures to higher, acute exposures.

An increase from pre- to 1 hr post-fumigation R/FR ratio mean levels also occurred for both the 120 and 220 mm^3m^{-3} ozone exposures significant at $0.10 > P > 0.05$

and $0.25 > P > 0.20$ respectively. The differences between these 1993 comparisons of pre- to 1 hr post-fumigation R/FR ratio means and the comparisons of the same R/FR ratio means for July and August, 1992, have separate explanations. For the July, 1992 measurements, the reduced numbers of stomata exposed to ozone may be the cause of an apparent lack of response at 1 hr post-fumigation. For the August, 1992 measurements, the ramets may have already suffered some damage or been under more stress than the ramets measured in July, 1992 or August, 1993. The ramets measured in August, 1992 were the same ones measured in July, 1992. The R/FR ratio mean for the pre-fumigation measurements in August, 1992 is significantly higher than for any of the other pre-fumigation R/FR ratio means ($P = 0.05$), and a pre-existing condition could have masked most of the response to the later fumigation.

The dramatic increases in R/FR ratio means at the 24 hour measurement seen in the August, 1992 and August, 1993 white pine data, and to a lesser degree in July, 1992, resulting from an increase in intensity in the red region of the spectrum (below 700 nm), may be caused by a reduction in chlorophyll levels (Lichtenthaler et al., 1986). Reflectance parameters such as the red edge inflection point, a good indicator of chlorophyll content (Rock et al., 1988; Vogelmann et al., 1993), showed no change that corresponded to the R/FR ratio mean increases at the 24 post-fumigation measurements in 1992 (Rock, unpublished data). Preliminary analysis of the 1993 reflectance data indicates similar results.

Alternatively, the increases in the R/FR ratios may be related to ozone effects on electron transport between PS II and PS I (Lichtenthaler et al., 1986). Increases in R/FR ratio mean levels may also be induced by ozone levels simply as a result of CO_2 concentrations falling and the photosynthetic rate decreasing due to rapid stomatal closure, causing excess energy to be "dumped", thermally and as fluorescence (Salisbury and Ross, 1985; Lawlor, 1990; Walker, 1992). This latter effect would most likely be seen during fumigation or shortly after, and recovery would be expected when the source

of ozone was removed, leading to reopening of the stomates (Schreiber et al., 1978). The suggestion of possible recovery at the 48 hour post-fumigation measurements may be an indication of this effect or, it may be a result of the plants' natural defenses against oxidants (Morre et al., 1990; Sen-Gupta et al., 1991). The increase in R/FR ratio means of the 1 hour post-fumigation measurements (August, 1993) may be due to interference with electron transport or the effects of rapid stomatal closure, with loss of chlorophyll less likely because of the time interval between the exposure and the 1 hr post-fumigation measurements.

Gas exchange data for all the ramets show no change in the photosynthetic rate related to ozone treatment for the 1992 short-term experiments (Eckert et al., 1993). Lack of response in gas exchange coupled with our measured increases in fluorescence R/FR ratios is consistent with the other studies, however. In studies of short-term exposure of beans to ozone, Schreiber et al. (1978) found evidence indicating that, initially, ozone does not directly affect PS II reaction centers, and that decreased quantum absorption by PS II appears unlikely. They also state that the sequence of electron transport from PS II through PS I and NADP to CO_2 appears unaffected by ozone at a time when water-splitting is severely curtailed. Lawlor (1990) states that, if water-splitting is blocked, plastoquinone (PQ) is not reduced but fluorescence increases. Fluorescence normally increases or decreases in direct rough proportion to the relative amount of PQ reduced. It should be further noted that white pine has been reported to return to photosynthetic rates statistically indistinguishable from pre-fumigation levels within one hour of ozone exposure cessation (Yang et al., 1983). Unlike DCMU damage, which blocks electron transport directly by occupying the Q_B site on the D_1 molecule of PSII chlorophyll, or damage done by ozone to rubisco or its catalytic activity, causing immediate changes in photosynthesis (Lehnherr et al., 1988; Dann and Pell, 1989), Coulson and Heath (1974) note that ozone may interfere with the normal pathway of energy flow by disrupting components of the thylakoid membrane without causing a

general disintegration of that membrane, and therefore may not cause immediate changes in photosynthesis.

The t values, probabilities, and significance levels for the comparisons between the pre- and 24 hrs post-fumigation R/FR ratio means compiled in Table 1 for these experiments suggest that a threshold response may exist in white pine similar to that shown for wheat by Farage et al. (1991). Because the August, 1993 220 mm^3m^{-3} ozone exposure produced a less significant R/FR ratio mean difference than the 120 or 180 mm^3m^{-3} ozone exposures, it may be suggested that a more rapid reduction in stomatal conductance occurs at the higher ozone concentration, resulting in a lower total uptake of ozone. This assumption appears to be at odds with the conclusion of Farage et al. (1991), that reduced conductance, in wheat, was the result of reduced photosynthetic capacity. However, the report of Yang et al. (1983) shows a greater maximum reduction of net photosynthesis within the first hour following exposure to higher ozone concentrations. If this results in a greater reduction of stomatal conductance, it seems reasonable to assume that less ozone will be taken into the mesophyll and assimilated, leading to less damage. If less damage occurs, then a return (recovery) to pre-fumigation R/FR values can be expected to begin sooner. The data presented in figure 8 may indicate that the trees fumigated with 220 mm^3m^{-3} ozone recovered to a greater extent at 48 hours after fumigation than those receiving the 120 mm^3m^{-3} ozone exposure.

FLD-R/FR ratios are derived from chlorophyll emissions at wavelengths corresponding to known atmospheric and solar absorption features. Table 2 provides a compilation of the t values, probabilities, and significance levels for the comparisons between the pre- and 24 hrs post-fumigation FLD-R/FR ratio means. The strong correspondence between results shown in Table 1 and Table 2 indicates that stress-induced fluorescence changes, measured with R/FR ratios, may be detectable in direct sunlight.

The results of this research are in agreement with the conclusions of previous

investigators that white pine does respond, physiologically, to the presence of ozone. Furthermore, the red/far-red ratios of chlorophyll fluorescence, derived from emission spectra measured in the steady state, can provide information, non-destructively, about the physiological responses of white pine subjected to short-term exposures of ozone; the Null Hypothesis is rejected. The fact that significant fluorescence changes were measured while no significant changes were seen in the reflectance or gas exchange data, indicates that fluorescence monitoring of vegetation in areas geographically and climatologically susceptible to the formation of ozone should be pursued. This monitoring could be accomplished with passive instrumentation that uses the sun as an excitation source, corresponding to the natural conditions under which ozone is most likely to be present and when plants are most likely to take ozone into their systems.

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TABLES

Table 1. The evaluation of the R/FR fluorescence ratio mean data comparisons between pre- and 24hrs post-fumigation measurements, showing the relative differences between the means of those two measurement times and their significance. The critical t values are from Lapin (1983).

Date	July 1992	August 1992	August 1993	August 1993
ozone (mm^3m^{-3})	180	180	120	220
df	15	15	5	5
$P_{R/FR}$	.10	.05	.05	.20
$t_{R/FR}$	1.75	5.50	2.88	1.67
$t_{P[df]}$	1.75	2.13	2.57	1.48
significance	90%	› 95%	› 95%	› 80%

Table 2. The evaluation of the FLD-R/FR fluorescence ratio mean data comparisons between pre- and 24hrs post-fumigation measurements, showing the relative differences between the means of those two measurement times and their significance. The critical t values are from Lapin (1983).

Date	July 1992	August 1992	August 1993	August 1993
ozone (mm^3m^{-3})	180	180	120	220
df	15	15	5	5
$P_{FLD-R/FR}$	.25	.05	.05	.10
$t_{FLD-R/FR}$	1.20	4.71	3.75	2.00
$t_{P[df]}$	1.20	2.13	2.57	2.02
significance	75%	› 95%	› 95%	‹ 90%

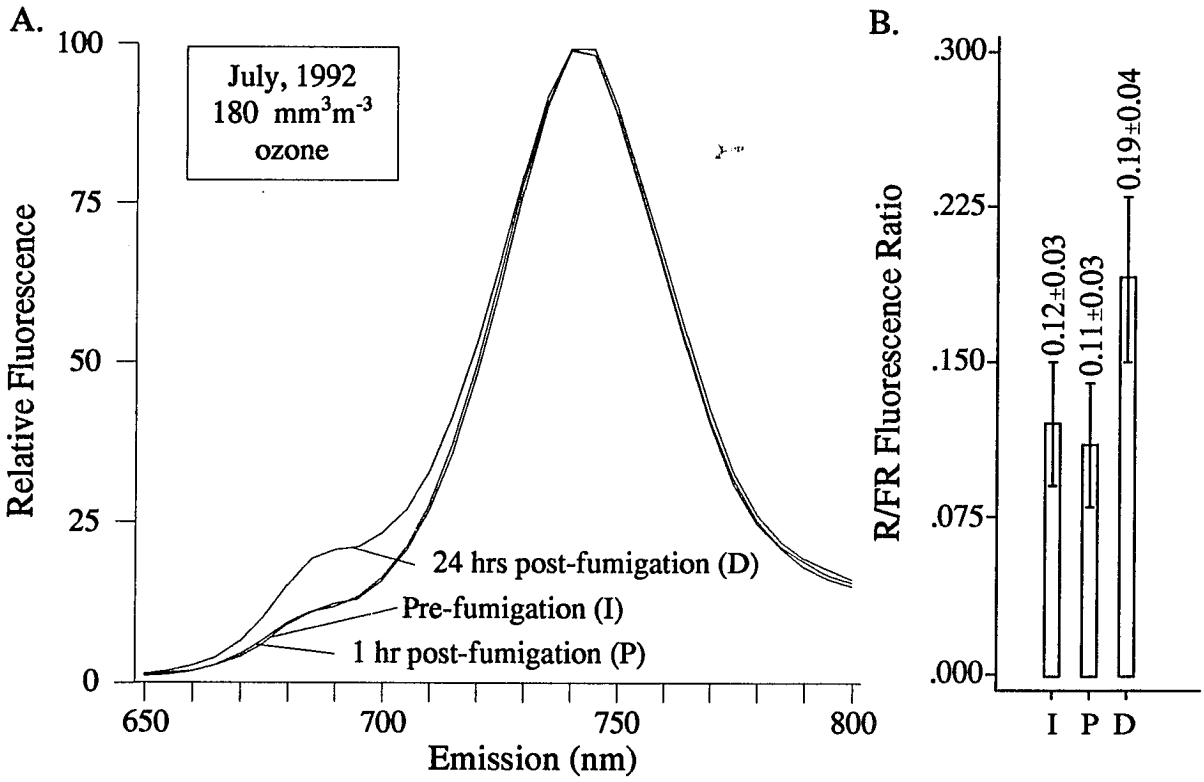

Figure 3. White pine ozone exposure at 180 mm^3m^{-3}, July, 1992, Acadia N.P., Maine.

(A.) Ozone exposure at 180 mm^3m^{-3}, mean fluorescence emission spectra (n = 16) for white pine ramets, with excitation at 470 nm, normalized to 100 at the spectral maximum, collected prior to (I), one hour after (P), and 24 hours after (D) a 3 hour fumigation, show an increase in the red region of the 24 hr post-fumigation mean spectrum.

(B.) The R/FR fluorescence emission ratio means and standard errors are presented in bargraph format. Although the spectral plot shows a clear separation of the 24 hrs post-fumigation R/FR ratio mean from those of the pre- and 1 hr post-fumigation R/FR ratio means, statistical analysis using the Student t test indicates that the difference between the pre- and 24 hrs post-fumigation R/FR ratio means is only significant at the 90% significance level ($P = 0.10$, $df = 15$, $t = 1.75$).

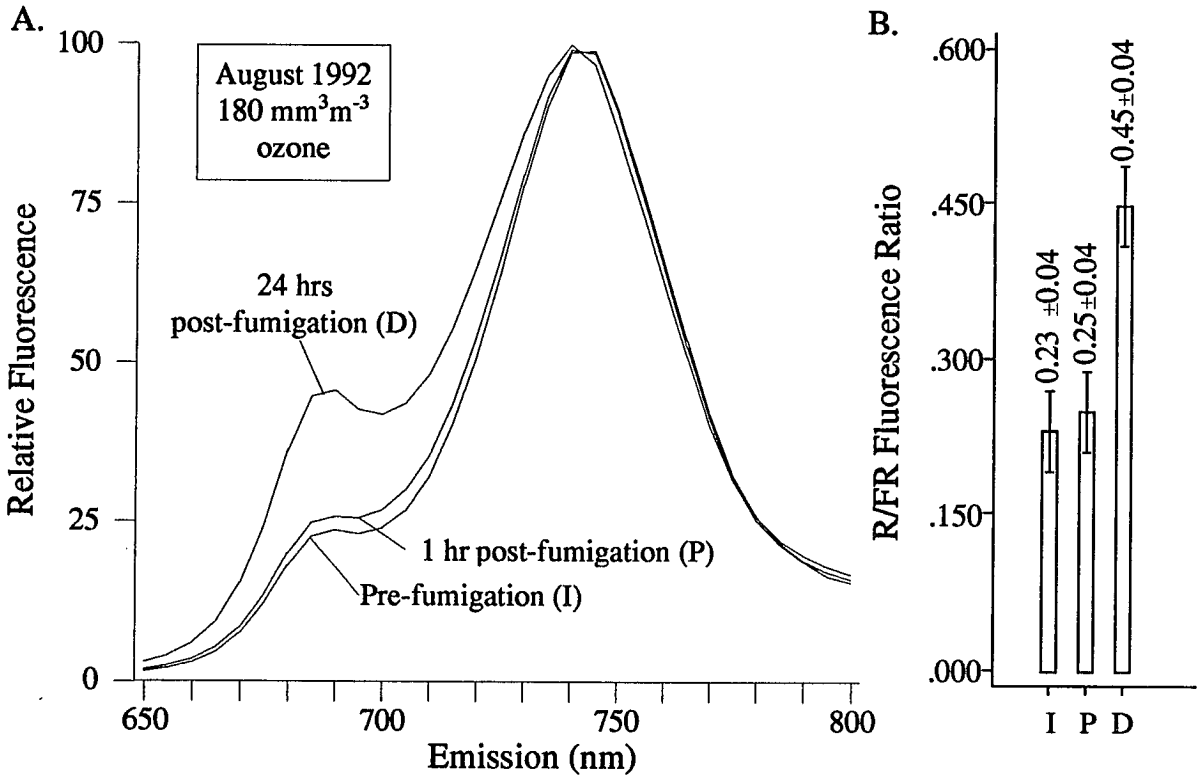

Figure 4. White pine ozone exposure at 180 mm^3m^{-3}, August, 1992, Acadia N.P., Maine.

(A.) Ozone exposure at 180 mm^3m^{-3}, mean fluorescence emission spectra (n = 16) for white pine ramets, with excitation at 470 nm, normalized to 100 at the spectral maximum, collected prior to (I), one hour after (P), and 24 hours after (D) a 3 hour fumigation shows a greater separation of the 24 hour post-fumigation measurement mean from the pre- and 1 hour post-fumigation means than was seen in the July spectral plot.

(B.) The R/FR fluorescence emission ratio means and standard errors are presented in bargraph format, and statistical analysis of the means shows that the difference between the pre-fumigation R/FR ratio mean the 24 hrs post-fumigation R/FR ratio mean is significant at greater than 95% (P = .05, df = 15, t = 5.50).

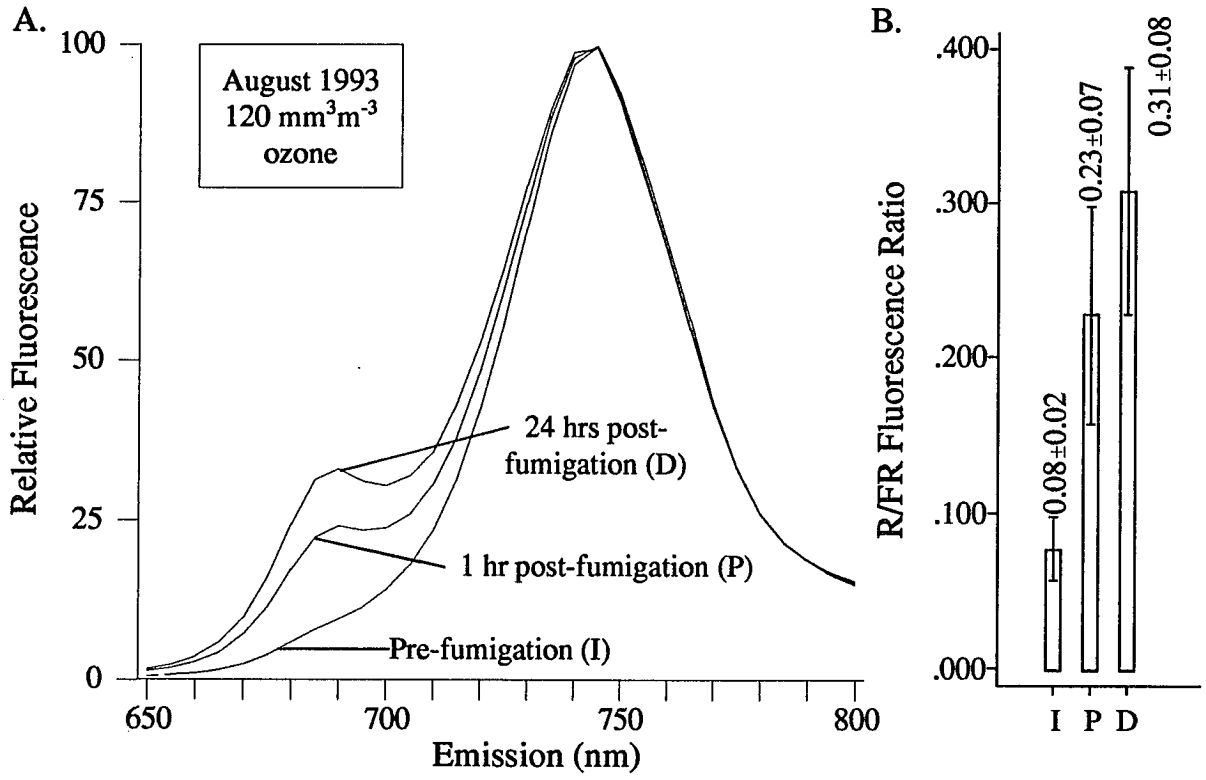

Figure 5. White pine ozone exposure at 120 mm^3m^{-3}, August, 1993, Acadia N.P., Maine.

(A.) Ozone exposure at 120 mm^3m^{-3}, mean fluorescence emission spectra (n = 6) for white pine ramets, with excitation at 470 nm, normalized to 100 at the spectral maximum, collected prior to (I), one hour after (P), and 24 hours after (D) a 3 hour fumigation are shown.
(B.) The R/FR fluorescence emission ratio means and standard errors are presented in bargraph format. Statistical analysis of the means shows that the pre-fumigation R/FR ratio mean differs from the 24 hrs post-fumigation R/FR ratio mean with a significance greater than 95% (P = .05, df = 5, t = 2.88). The 1 hr post-fumigation R/FR ratio mean is greater than the pre-fumigation R/FR ratio mean with a significance from 90% to 95% (.10 › P › .05, df = 5, t = 2.14).

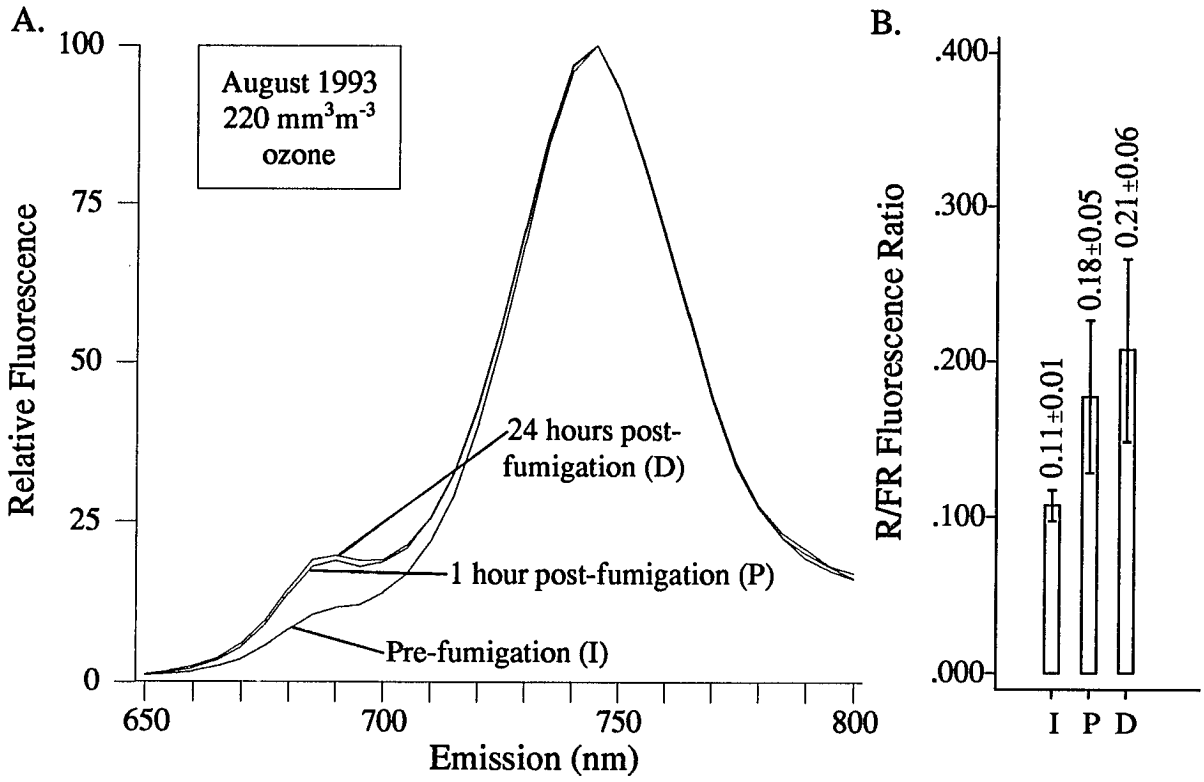

Figure 6. White pine ozone exposure at 220 mm^3m^{-3}, August, 1993, Acadia N.P., Maine.

(A.) Ozone exposure at 220 mm^3m^{-3}, mean fluorescence emission spectra (n = 6) for white pine ramets, with excitation at 470 nm, normalized to 100 at the spectral maximum, collected prior to (I), one hour after (P), and 24 hours after (D) a 3 hour fumigation are displayed.

(B.) The R/FR fluorescence emission ratio means and standard errors are presented in bargraph format. Statistical analysis of the means shows that the 24 hrs post-fumigation R/FR ratio mean is greater than the pre-fumigation R/FR ratio mean with a significance of only 80% (P = .20, df = 5, t = 1.67). The difference between the pre-fumigation R/FR ratio mean and the 1 hr post-fumigation mean is significant from 75% to 80% (.25 › P › .20, df = 5, t = 1.40).

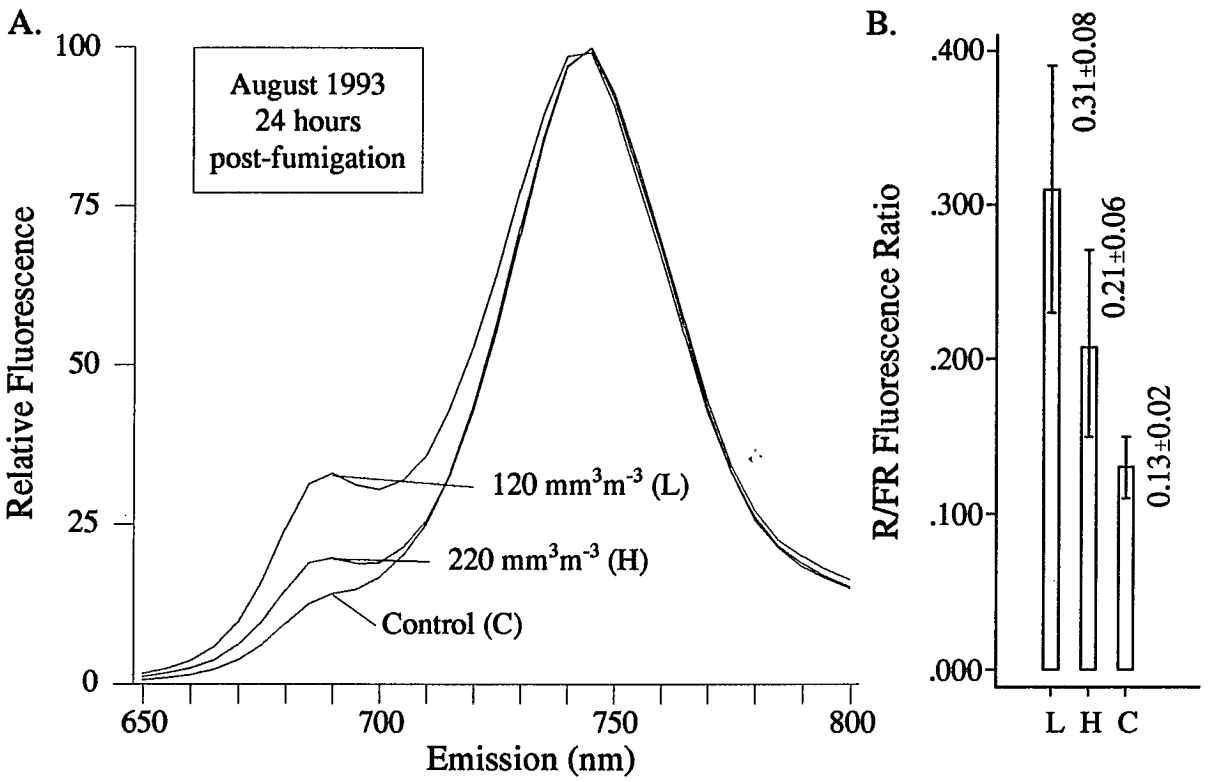

Figure 7. White pine at 24 hrs post-fumigation, August, 1993, Acadia N.P., Maine.

(A.) Twenty four hours post-fumigation mean fluorescence emission spectra (n = 6) for white pine, with excitation at 470 nm, normalized to 100 at the spectral maximum, of the ramets treated with 120 mm^3m^{-3} ozone (L) and 220 mm^3m^{-3} ozone (H) and the untreated ramets, control (C) are presented.

(B.) The means and standard errors of the R/FR fluorescence emission ratios are shown in bargraph format. Statistical analysis of the means shows that the R/FR ratio mean for the control ramets lies significantly below the R/FR ratio mean for the 220 mm^3m^{-3} ozone treated ramets (P = .05, df = 5, t = 4.00).

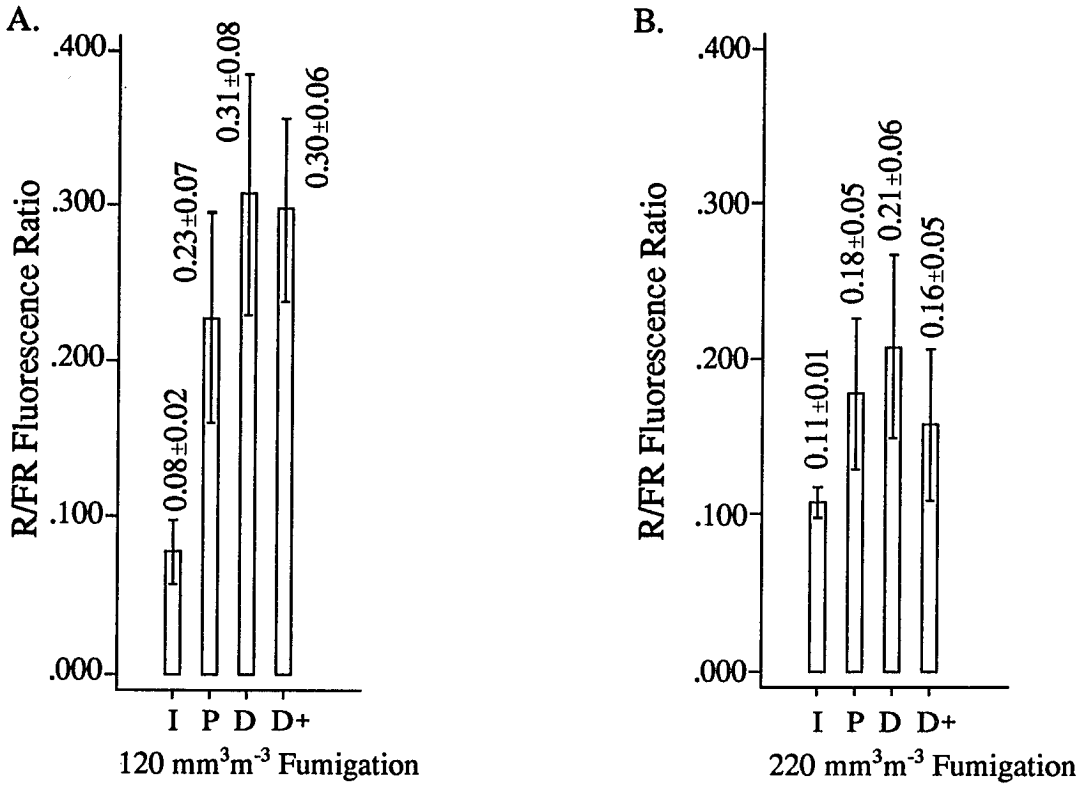

Figure 8. Red/far-red ratio mean comparisons, including 48 hr post-fumigation.

(A.) August, 1993 R/FR fluorescence ratio mean values (n = 6) and standard errors are shown for measurements made of white pine. The excitation is 470 nm, and the data were collected prior to (I), one hour after (P), 24 hours after (D), and 48 hours after (D+) a 3 hour fumigation of the ramets with 120 mm^3m^{-3} ozone.

(B.) The R/FR fluorescence emission ratio means (n = 6) and standard errors are shown for measurements made of white pine ramets exposed to 220 mm^3m^{-3} ozone, also in August 1993. The statistics for these data show that there is no significant difference between the 24 hrs post-fumigation R/FR ratio mean and the 48 hrs post-fumigation R/FR ratio mean for 120 mm^3m^{-3} exposure (P = .80, df = 5,t = .17), but at 220 mm^3m^{-3} ozone exposure there is a slight decrease from the 24 hrs post-fumigation R/FR ratio mean to the 48 hrs post-fumigation R/FR ratio mean (P = .25, df = 5, t = 1.00).

CHAPTER II[3]

STEADY-STATE CHLOROPHYLL FLUORESCENCE DIFFERENCES IN BEANS (*Phaseolus vulgaris* L.) GROWN IN CADMIUM DOPED SOIL[1]

ABSTRACT

In July, 1993 five replicates of beans (*Phaseolus vulgaris* L.) were grown in pH 4.9 soil doped with cadmium (Cd, 0 to 50 mg Kg^{-1}). Steady-state far-red fluorescence maxima (FRmax) strongly correlated with Cd dose (r = 0.96) and dry weight (r = 1.0). A significant dry weight gradient was produced (r = 0.95). Differences in red/far-red fluorescence ratios (R/FR) among the Cd treatments were not significant (P = 0.05). Chlorophyll levels, a controlling factor for R/FR, were also not significantly different (P = 0.05). When the experiment was repeated in April, 1994, FRmax strongly correlated with Cd dose (r = 0.97) and dry weight (r = 0.91), and a strong correlation between dry weight and Cd dose was also noted (r = 0.95). However, the second experiment produced a strong correlation between R/FR and Cd dose (r = 0.91) and a high correlation between R/FR and dry weight (r = 0.86). Chlorophyll levels were significantly different among the treatments (P = 0.05), strongly correlated with R/FR (r = 0.99) and Cd dose (r = 0.95), and well correlated with dry weight (r = 0.90). These results indicate that stress to beans grown in cadmium contaminated soil can be detected with steady-state R/FR fluorescence ratio differences when that stress results in pre-visual loss of chlorophyll, and suggest that steady-state far-red fluorescence intensities can be used to detect stressed conditions before chlorophyll is lost.

3/ This Chapter was submitted for publication in *Plant Physiology* 20 October 1994.

INTRODUCTION

In the lithosphere, cadmium is a rare element, sixty seventh in order of abundance and found in concentrations of 0.1 to 0.2 μg/g. Yet, because of extremely useful properties developed since its discovery by F. Strohmeyer in 1817, we have extracted it from sulfide ores and concentrated it to high purity. The many mineral forms into which cadmium can be converted appear in products that surround us, literally, every day of our lives. These products include antiseptics, fungicides, lubricants, colorings and pigments for porcelain, glass, cloth, photography, fireworks and house paints, the phosphors used in color televisions and computer monitors, solar energy cells, and the batteries that power our cameras, mobile phones, and portable computers. Many peoples lives have been improved and even saved through the use of cadmium in such products as the low melting point alloys in automatic fire sprinkler systems and the photoelectric cells of smoke detectors. Cadmium is also used to control nuclear fission reactions in use in many power plants that supply a significant portion of the world's electricity that might otherwise be generated with fossil fuels.

For all the positive aspects of cadmium use there is also a down side. Cadmium is highly toxic to humans (Yasumura et al., 1980) and while it is readily taken up by plants, has no essential purpose nor serves any metabolic function in plants (Jastrow and Koeppe, 1980; Rudd, 1987; Punz and Sieghardt, 1993), it can be toxic to them as well. Yasumura et al. (1980) report the symptoms of cadmium consumption and inhalation, and note, without numbers, that most cases of the former have not been fatal and that the latter is suffered by the naive, careless or untrained worker where cadmium is extracted or processed. The sole incident of chronic ingestion of cadmium from the normal consumption of food was the disease named Itai-itai (ouch-ouch) that, until 1965, involved an estimated 200 Japanese, over half of whom succumbed to the toxicity (Rudd, 1987).

To understand the potential for harm to humans from cadmium in the food chain,

extensive research was begun in 1970 (Holmgren et al., 1993). The dietary model for the United States presented by the Food and Drug Administration estimated that teen-aged males ingested from 20 to 51 μg per day. These levels were considered to be too close to the World Health Organization recommended acceptable intake of 52 to 71 μg per day for 60 Kg humans. However, a better understanding of the potential hazard is presented by Holmgren et al. (1993) in the results of a long term study of the major crop producing areas of the United States. The conclusions they reached are that the data show little evidence of significant accumulation of cadmium in cropland soils and that the original concern about risk from soil cadmium was overstated. It should be noted that the sites in the study specifically avoided areas of known anthropogenic contamination not related to normal agricultural fertilization practices and areas where the application of waste sludge was conducted.

A small portion of the multitude of uses to which cadmium can be applied was listed above, and because of the "throw away" nature of our society, it is easy to see how quickly cadmium can build up in the sewage stream. However, cadmium is readily removed from the effluent and concentrated in the remaining sludge (Rudd, 1987). When this sludge is converted to a finished compost, field trials have shown little difference in plant uptake as compared to plants grown without sludge compost (Henry and Harrison, 1992). Because the availability of cadmium is dependent upon soil pH (Jastrow and Koeppe, 1980), greater vigilance would be required in monitoring the application of sludge compost in the eastern part of the United States where soil pH is generally lower (4.5 to 5.9) than in the Great Plains (6.9) and Southwest (7.6) where soil pH is generally higher (Holmgren et al, 1993).

In 1989 the Office of Environment Restoration and Waste Management (EM) was established to deal with waste previously and currently generated from nuclear weapons production and testing (David, 1993). Activity in 34 states has left a cleanup responsibility that covers many different potential contaminants including organic

compounds, inorganic materials, and transuranic and other radioactive elements. Cadmium is but one of the several inorganic materials that is of interest to the EM in its pursuit of the detection, location, and quantification of contaminants in vegetation, soils and ground water. The methods and results described in this report are directed toward a better understanding of the manner in which fluorescence changes in vegetation subjected to cadmium uptake can be detected with laboratory techniques that will provide information relative to the use of passive remote sensing instruments that make use of techniques, such as the Fraunhofer line depth method (McFarlane et al, 1980; Carter et al., 1990).

MATERIALS AND METHODS

Experiments conducted at the University of New Hampshire in 1993 and 1994 involved the growing of bean plants (*Phaseolus vulgaris L.*) in a greenhouse in soil doped with pre-determined levels of cadmium. Low pH forest soil (4.9), used to ensure the mobility of cadmium cations, was screened, thoroughly blended with additions, and distributed to 6 in. pots. A cadmium chloride ($CdCl_2$) solution was prepared to provide 5 replicates of 5 treatments at 10, 20, 30, 40, and 50 mg Kg^{-1} Cd compared to the weight of soil found in a single pot plus a control of 0 mg Kg^{-1} Cd. Because the soil was deficient in magnesium and raising the pH was not desirable, Epsom salt, at 600 mg Kg^{-1}, was added to the soil in each pot. An initial fertilization of the soil was provided with the inclusion of 250 mg Kg^{-1} nitrogen, phosphorus, potassium (NPK) 20:20:20 per pot, and a twice weekly application of NPK 20:20:20 at 24 mg Kg^{-1} was continued until the third trifoliate leaf was fully expanded to ensure that the root mass had enough time to gain access to as much of the Cd contaminated soil as possible. Emission spectral measurements were taken with a fluorescence spectrophotometer. The two lateral lobes of the third trifoliate leaf were measured and a 1 cm^2 portion was removed for chlorophyll analysis. The

above-ground portion of each plant was harvested and the fresh weight was measured. Plants were then oven dried at 70° C for a minimum of 24 hrs and measurements were made of dry weights.

Fluorescence Spectroscopy

A fluorescence spectrophotometer, Perkin-Elmer MPF-44B[2], has excitation and emission scanning monochromators with a measuring range from 200 to 900 nm. Adjustable slit widths accommodate bandwidth limiting, and a ratio mode is used to minimize the effects of any potential drift in the excitation source (a 150 watt Xenon lamp powered by a current-stabilized supply). The signal detectors are Hamamatsu R928 photomultipliers. A light excluding extension was added to the sample compartment to accommodate whole potted plants. For these experiments, emission intensity levels were collected at 5 nm increments, from 650 to 800 nm with an excitation wavelength of 470 nm. Intensity levels were corrected for the response characteristics of the instrument excitation source, photodetector, and excitation and emission optics that vary with wavelength. The emission spectra thus produced provided the data from which red/far-red (R/FR) fluorescence ratios were derived for analysis.

Chlorophyll extractions were made with dimethyl sulfoxide (DMSO) after Hiscox and Israelstam (1979), and the calculations of pigment concentration were based on formulas presented by Lichtenthaler (1987) for 80% acetone following Hiscox and Israelstam's use of Arnon's formulas for 80% acetone. The process was straight forward: a leaf sample (1 cm^2), collected from a leaf lobe so as to include the area of fluorescence measurement, was placed in a vial with 7 ml of DMSO and incubated at 65° C until the leaf material no longer retained any color. The leaf material was removed from the solution which was then brought to 10 ml by adding more DMSO. An aliquot of the solution was measured immediately with a Beckman DU/7 absorption spectrophotometer

and the remainder was stored at around 4° C to satisfy any potential need for further analysis.

RESULTS

Thirty five days after planting in July, 1993, data was gathered from 30 bean plants grown in soil doped with cadmium doses of 0 to 50 mg kg^{-1}. Mean fluorescence emission spectra (n = 10) are shown in figure 9. Mean dry weights of above ground growth produced a gradient that was strongly correlated (r = 0.95) with cadmium dosage (figure 10). During the analysis of the spectral information, the data for the 40 mg kg^{-1} treatment was judged to be anomalous due to unknown causes, but is included here for completeness. The mean R/FR fluorescence ratios showed no significant increase (P = 0.05) with increased cadmium dosage (data not shown). The chlorophyll content of samples removed from the leaves at the time of the fluorescence measurements also showed no significant differences among the treatments and no correlation with either the cadmium dosages or plant dry weights (data not shown). However, the general increase in the mean fluorescence intensities in the far-red (ca 735 nm, figure 9) produced a high correlation with cadmium dosage (r = 0.96) when the anomalous 40 mg kg^{-1} treatment was ignored (figure 11). An even stronger correlation was seen between the mean far-red fluorescence maxima and the mean dry weights (r = 1.0, figure 12).

A second set of beans were planted in April, 1994 using the same soil type, pot sizes, nutrient additives, and cadmium treatments as before, with data collected for fluorescence, dry weights, and chlorophyll content after 39 days of growth. The mean fluorescence emission spectra (n = 10) displayed in figure 13 show a general increase in fluorescence intensity with cadmium treatment similar to that noted for the beans measured in 1993. Dry weights for plant above ground growth again produced a strong correlation (r = 0.95) with cadmium dosage (see figure 14). The mean far-red

fluorescence maxima were also strongly correlated with both cadmium dosage and mean plant dry weights ($r = 0.97$ and $r = 0.91$, respectively in figures 15 and 16). Unlike the plants grown in 1993, mean R/FR fluorescence ratios for the 1994 beans showed strong differences among treatments (i.e. a One-Way ANOVA with 5/54 degrees of freedom produced an F value of 18.68 versus a Table F value of 2.38 at $P = 0.05$), a high correlation with cadmium dosage ($r = 0.91$) and a good correlation ($r = 0.86$) with mean plant dry weights (figures 17 and 18). Measurements of the chlorophyll content of samples from each individual leaf lobe showed significant differences among all the treatments (i.e. a One-Way ANOVA with 5/54 degrees of freedom produced an F value of 10.12), strong correlations with mean R/FR fluorescence ratios ($r = 0.99$) and cadmium dosage ($r = 0.95$), and a high correlation with mean plant dry weights ($r = 0.96$). The data for these analyses are shown in figures 19, 20 and 21).

DISCUSSION AND CONCLUSIONS

Red/far-red fluorescence ratios can be used to detect different levels of stress in bean plants due to cadmium contamination when that stressing factor affects chlorophyll content. The strong correlations between chlorophyll contents and R/FR fluorescence ratios for the April, 1994 plants, and the lack of any differences in either chlorophyll contents or R/FR fluorescence ratios among treatments for the July, 1993 plants is in direct agreement with the conclusions of Lichtenthaler et al. (1986) about the relationship between R/FR fluorescence ratios and chlorophyll content. The overall fluorescence increases seen in the July, 1993 measurements may be an indication of stress even when chlorophyll content is not affected. Krupa et al. (1993) found that while photochemical efficiency of photosystem II (PSII) measured with dark adapted plants remained stable over a range of cadmium dosages, measurements of photochemical efficiency in the steady-state declined. They state that this implies an inhibition of energy consumption by

cadmium without an accompanying reduction in photochemistry. Their findings correspond with our July, 1993 results where fluorescence in general increased (i.e. reduced energy consumption) but the R/FR ratios did not (i.e. no change in photochemistry).

The cadmium treatments in both experiments reported here produced obvious size differences in the bean plants in agreement with most previous studies (Jastrow and Koeppe, 1980) and a reduction in chlorophyll content in the April, 1994 beans, but did not produce visible differences in the intensity or shades of green among the leaves for either experiment. While uptake of cadmium is dependent on the concentration in soil (Turner, 1973) and soil pH (Reber, 1989), and chlorophyll levels have been shown to be reduced in bean seedlings grown only in solutions of cadmium acetate (Padmaja et al., 1990), the greatest amount of cadmium taken up by plants is retained in the roots (Narwal et al., 1990).

The results of the 1993 cadmium study are in agreement with findings of Krupa et al. (1992) who found significantly reduced growth with increased cadmium dosage in beans grown in Hoagland solution, with Turner (1973) who found no change in fluorescence yields, and Murata et al. (1970) who noted no significant effects on fluorescence yields from isolated spinach chloroplasts doped with concentrations of cadmium below 5 mM. On the other hand, the 1994 results agree with the information provided by Van Duijvendijk-Matteoli and Desmet (1975) that cadmium at a concentration of 15 mM was found to inhibit electron transport on the electron donor side and action site of PSII. The electron transport inhibition would result in increased R/FR fluorescence ratios (Lichtenthaler and Rinderle, 1988). Padmaja et al. (1990) noted reduced chlorophyll levels that would also produce increased R/FR fluorescence ratios (Lichtenthaler et al., 1986).

The difference in responses to cadmium for the plants in the two experiments may be that, while the variables under direct control of the investigators (e.g. soil type, soil

quantity, nutrient additives, and cadmium concentration) were the same for both, growing conditions, even in the greenhouse, differed with the times of the year (i.e. temperature ranges and hours of natural light). That these parameters would have a major effect on the cadmium concentration in the leaves that would determine chlorophyll levels and therefore R/FR fluorescence ratios is a matter for further investigation.

Monitoring of fluorescence at wavelengths corresponding to known atmospheric and solar absorption features could be accomplished with passive instrumentation, such as a Fraunhofer line discriminator (FLD) that uses the sun as an excitation source (McFarlane et al, 1980; Carter et al., 1990). FLD-R/FR fluorescence ratios can be derived from the laboratory collected chlorophyll emission spectra at these same wavelengths (Theisen et al., 1994). The FLD-R/FR fluorescence ratios have been found to agree well with R/FR fluorescence ratios calculated from the red and far-red maxima. The conditions under which FLD measurements are made are also those under which plants are most likely to be attempting to function at their optimum level and therefore reveal the consequences of any stressing factors to which the plant is subjected.

ACKNOWLEDGMENTS

Special thanks are due Dr. David L. Campbell, Branch of Geophysics, U.S. Geological Survey for the loan of the fluorescence spectrophotometer, Drs. George Estes and Owen Rogers, Plant Biology Dept., and the staff of the green house and Kingman Farm for all their assistance.

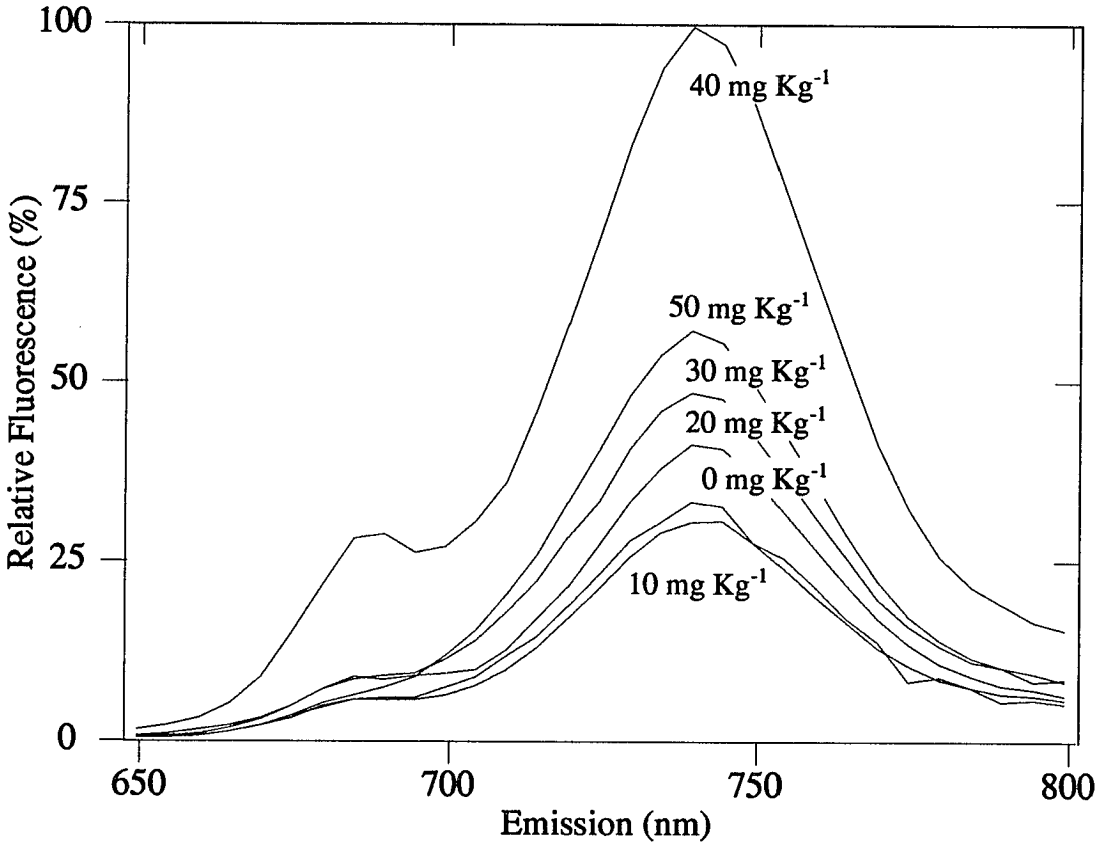

Figure 9. Emission spectra of beans planted in July, 1993.

The beans were measured after 35 days, produced mean fluorescence emission spectra shown normalized (n = 10). The beans were grown in soil contaminated with predetermined levels of cadmium. The excitation wavelength was 470 nm.

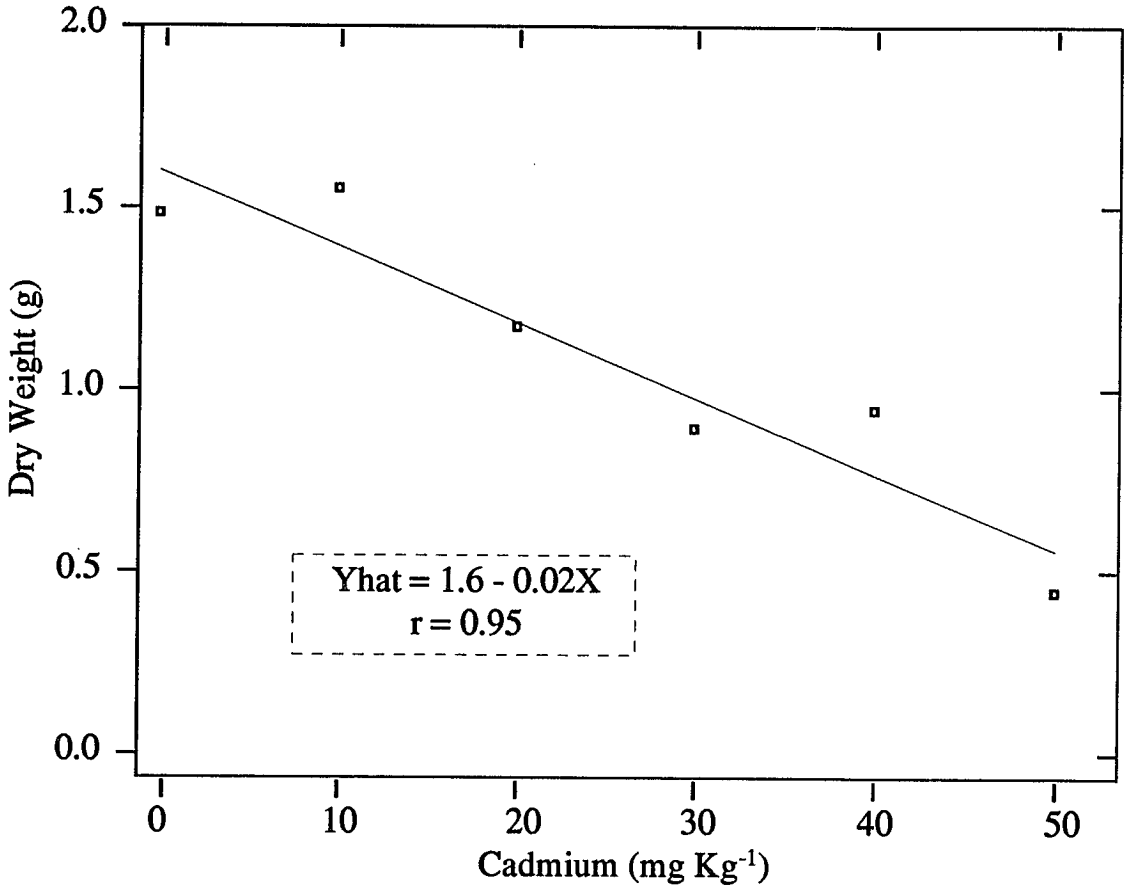

Figure 10. Cadmium vs dry weight for beans planted July, 1993.

The beans were grown in soil contaminated with predetermined levels of cadmium. Correlations between the mean dry weights of above ground growth (n = 5) and cadmium dosage are shown with the least squares fit line.

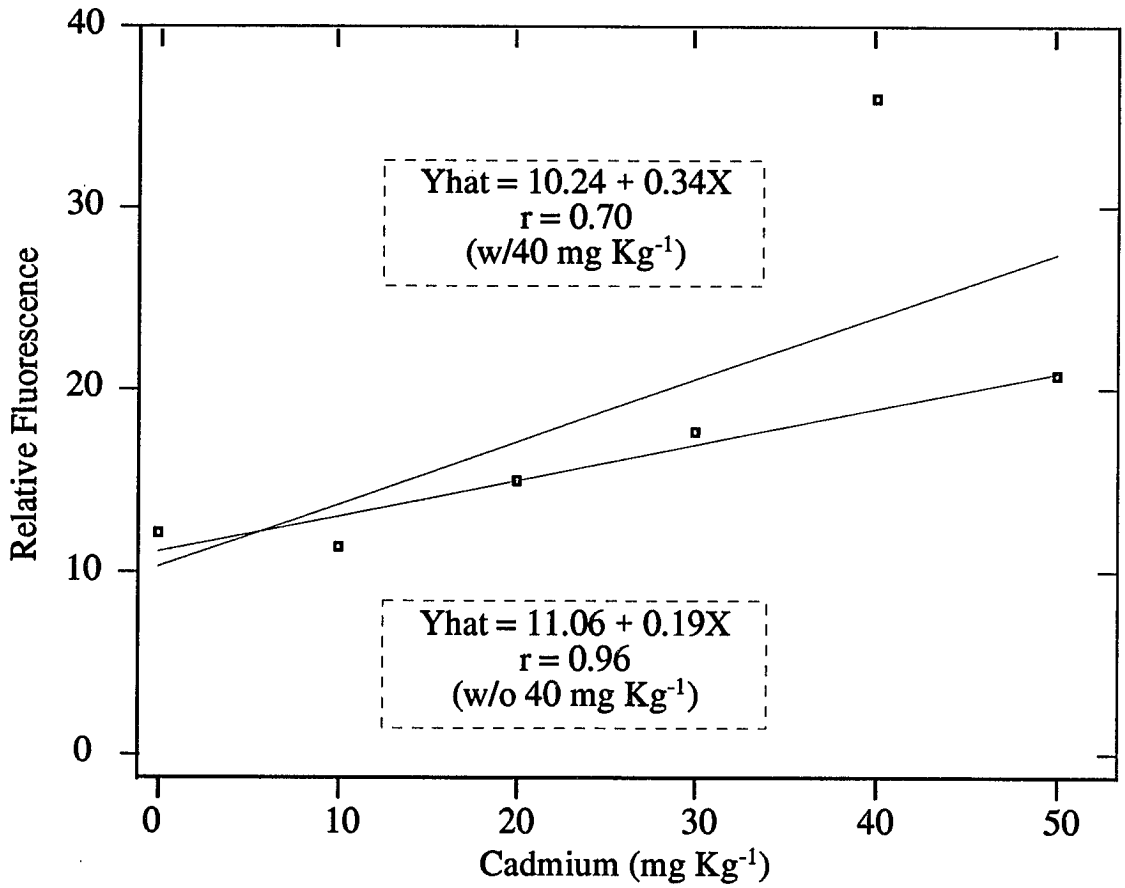

Figure 11. Cadmium vs far-red fluorescence maxima for beans planted July, 1993.

The beans were grown in soil contaminated with predetermined levels of cadmium. Correlations between the mean far-red fluorescence maxima (n = 10) and cadmium dosage are shown with the least squares fit line.

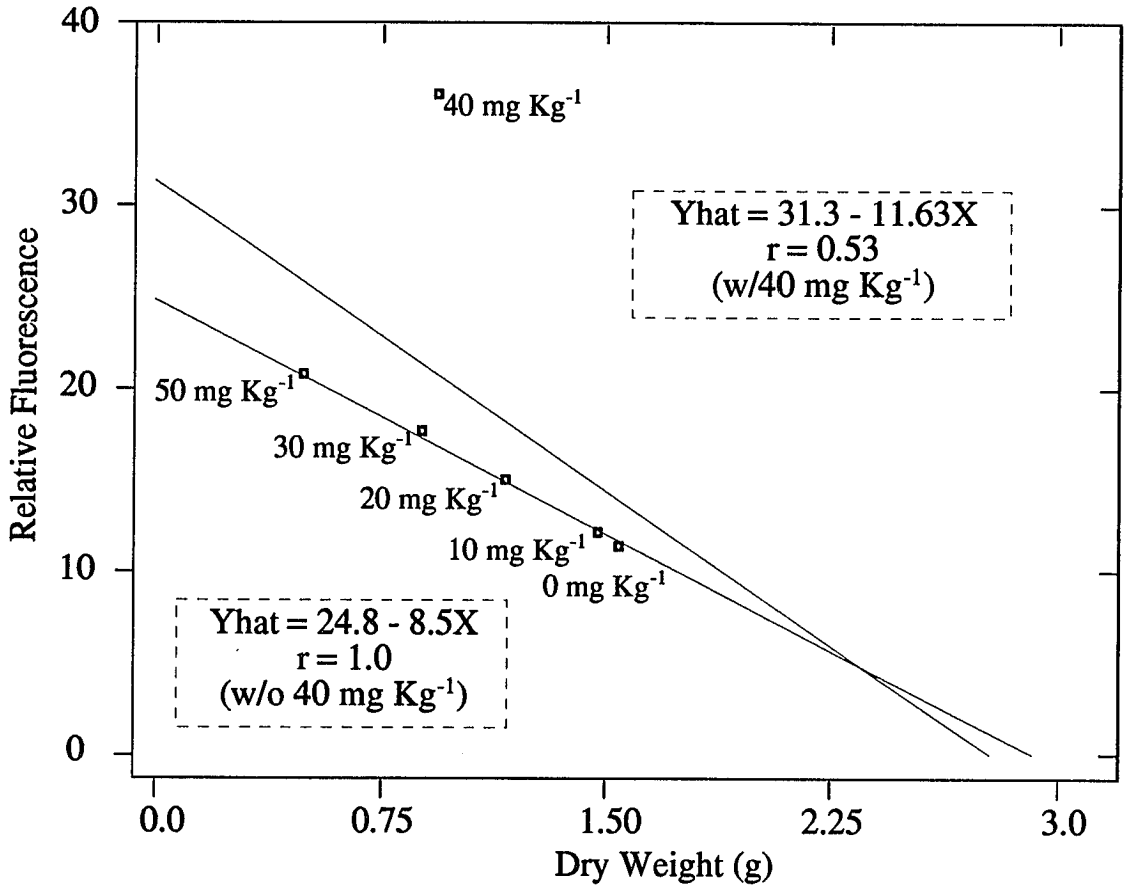

Figure 12. Dry weight vs far-red fluorescence maxima for beans planted July, 1993.

The beans were grown in soil contaminated with predetermined levels of cadmium. Correlations between the mean far-red fluorescence maxima and the mean dry weights of above ground growth are shown with the least squares fit line.

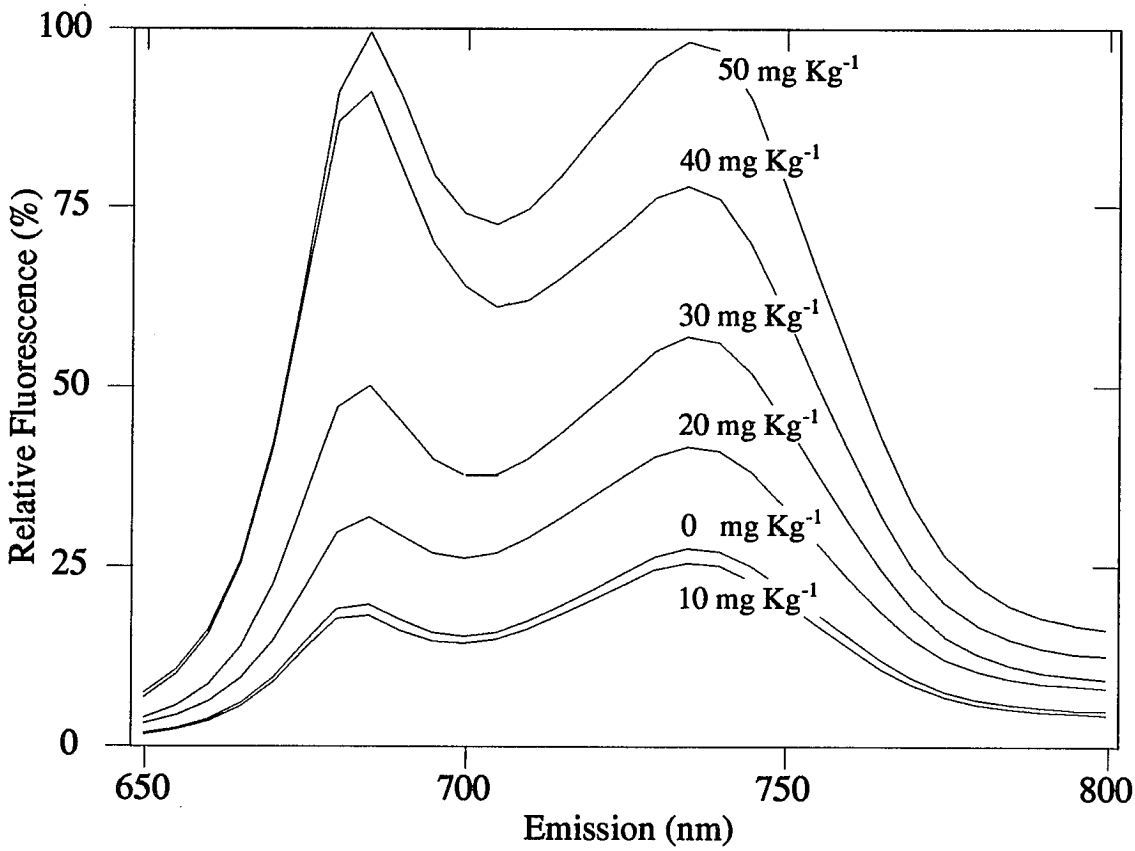

Figure 13. Emission spectra of beans planted in April, 1994.

The beans were measured after 39 days, produced mean fluorescence emission spectra shown normalized (n = 10). The beans were grown in soil contaminated with predetermined levels of cadmium. The excitation wavelength was 470 nm.

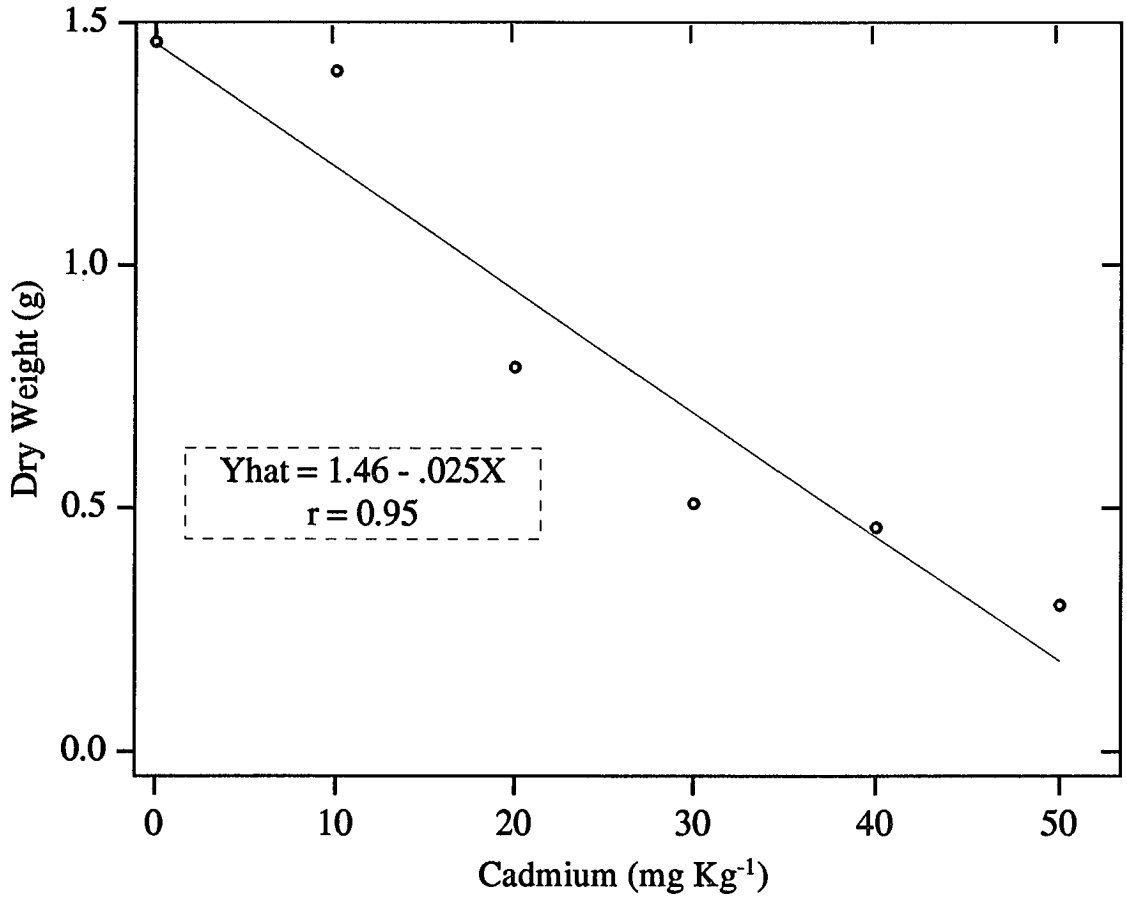

Figure 14. Cadmium vs dry weight for beans planted in April, 1994.

Correlations are shown with the least squares fit line (n = 5), and the data points are labeled with the cadmium dasage.

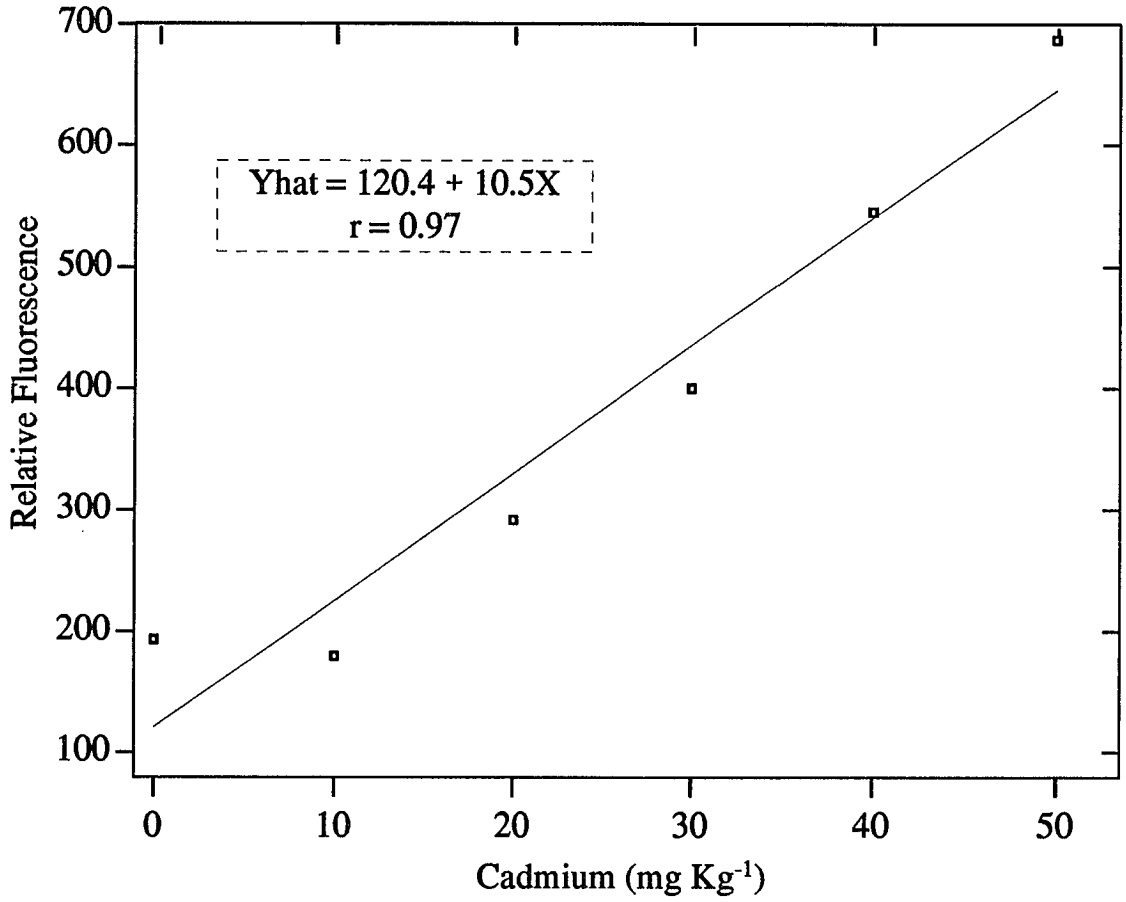

Figure 15. Cadmium vs far-red fluorescence maxima for beans planted in April, 1994.

Correlations are shown with the least squares fit line (n = 10), and the data points are labeled with the cadmium dasage.

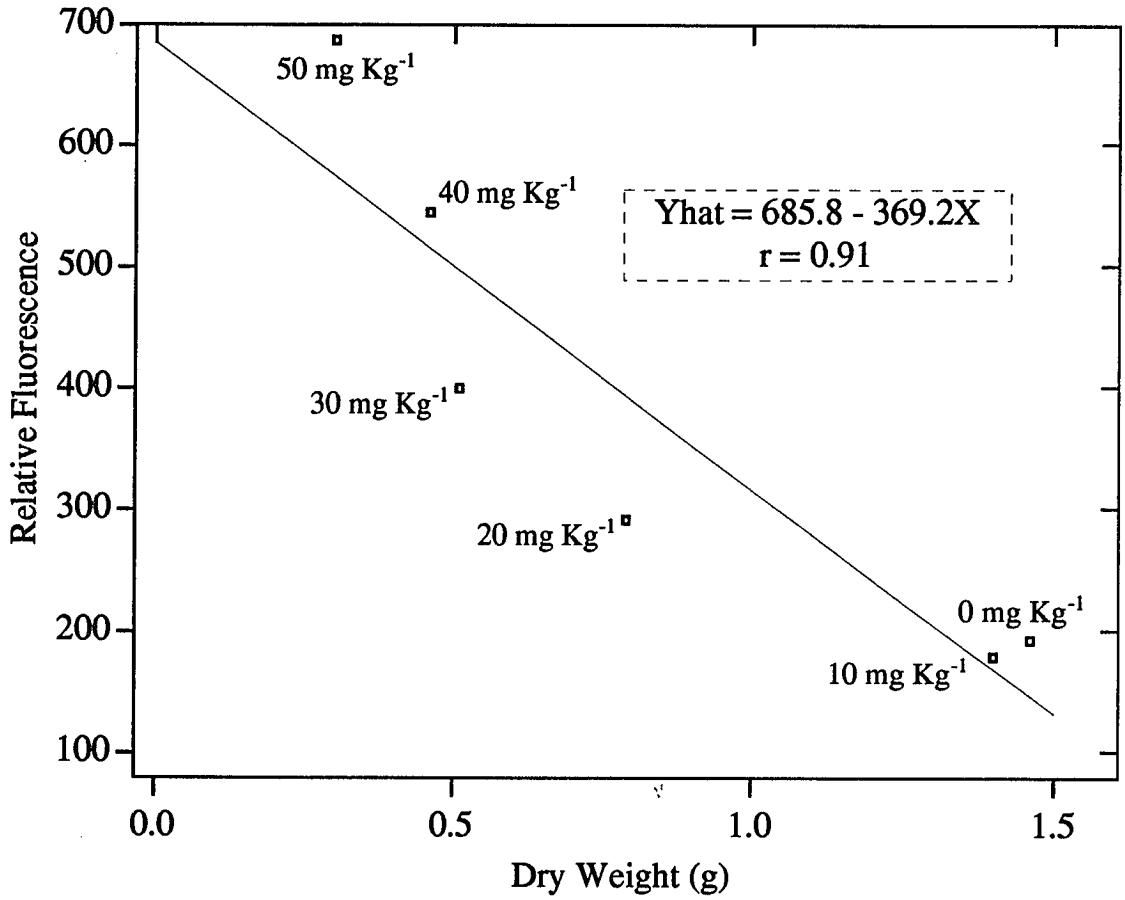

Figure 16. Dry weight vs far-red fluorescence maxima for beans planted in April, 1994.

Correlations are shown with the least squares fit line, and the data points are labeled with the cadmium dasage.

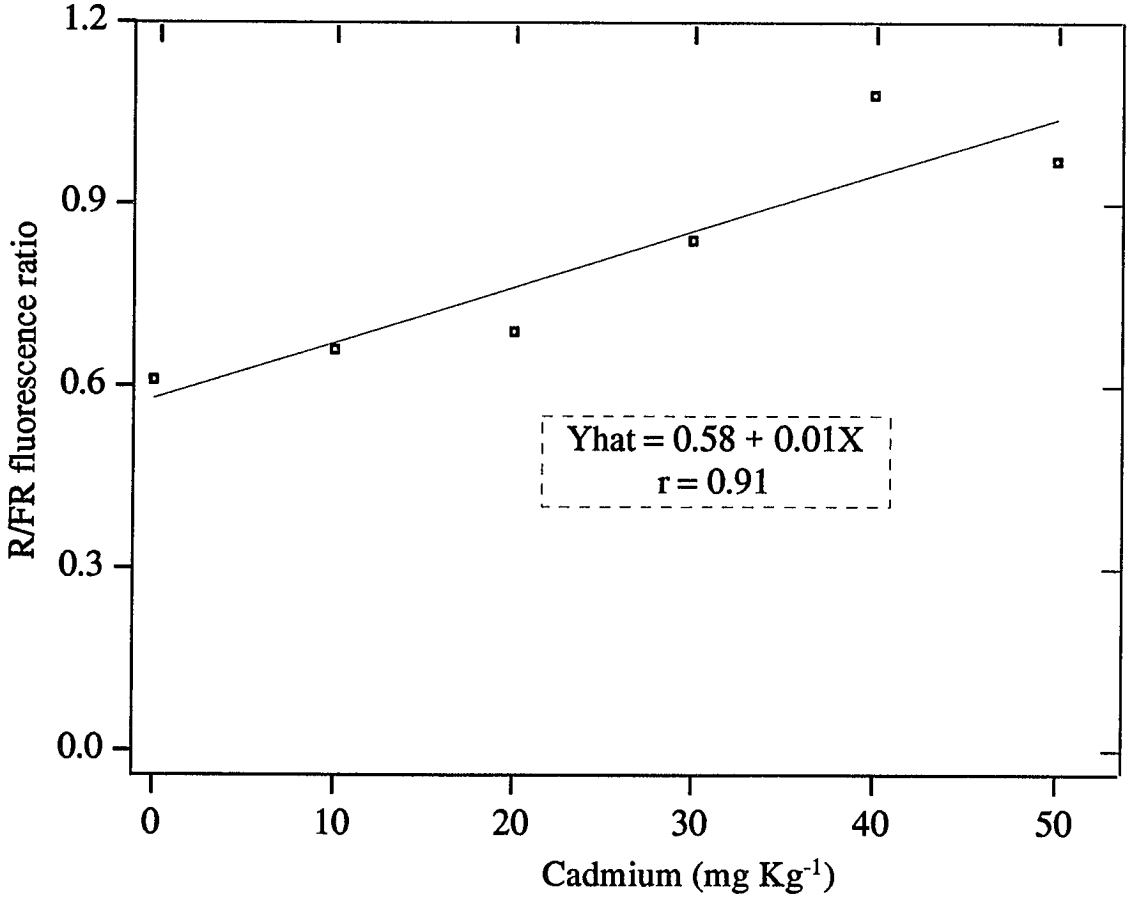

Figure 17. Cadmium vs mean R/FR fluorescence ratios for beans planted in April, 1994.

Correlations are shown with the least squares fit line (n = 10), and the data points are labeled with the cadmium dasage.

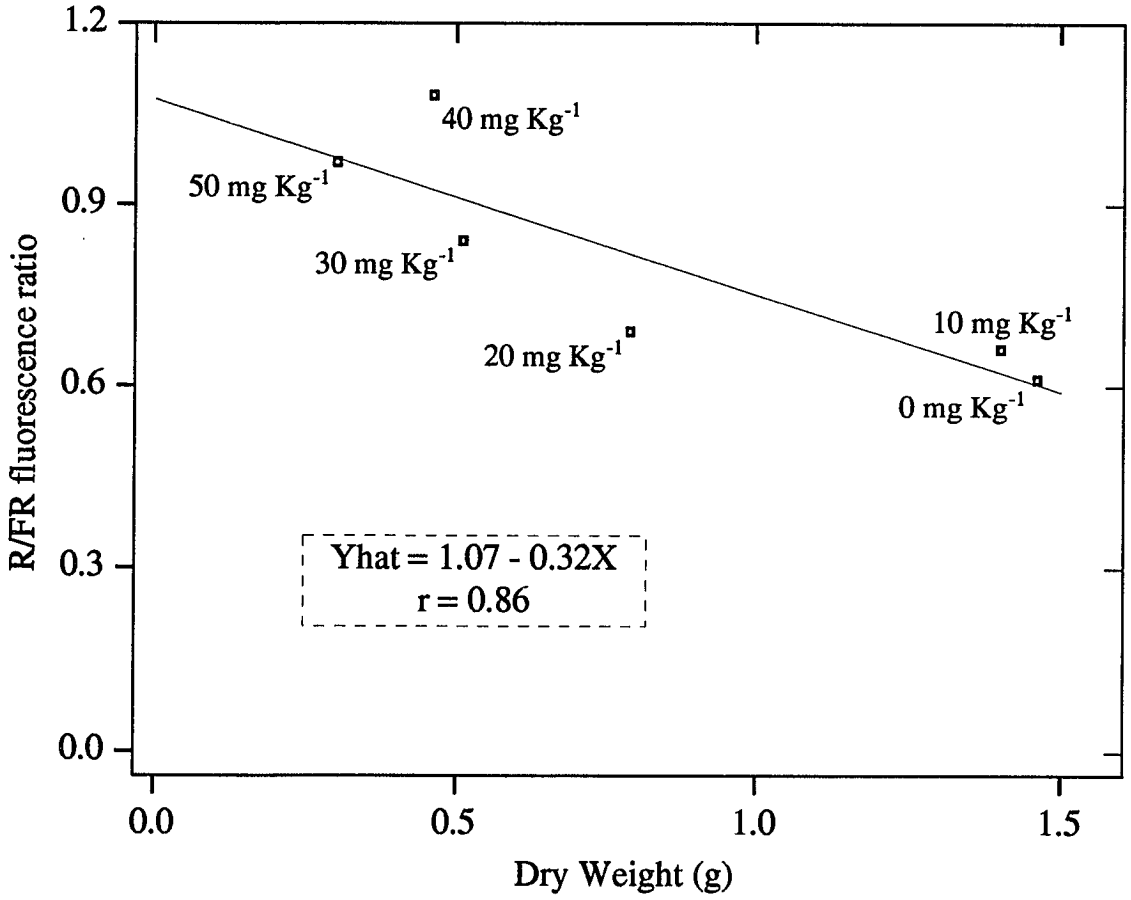

Figure 18. Dry weight vs mean R/FR fluorescence ratios for beans planted in April, 1994.

Correlations are shown with the least squares fit line (n = 5), and the data points are labeled with the cadmium dasage.

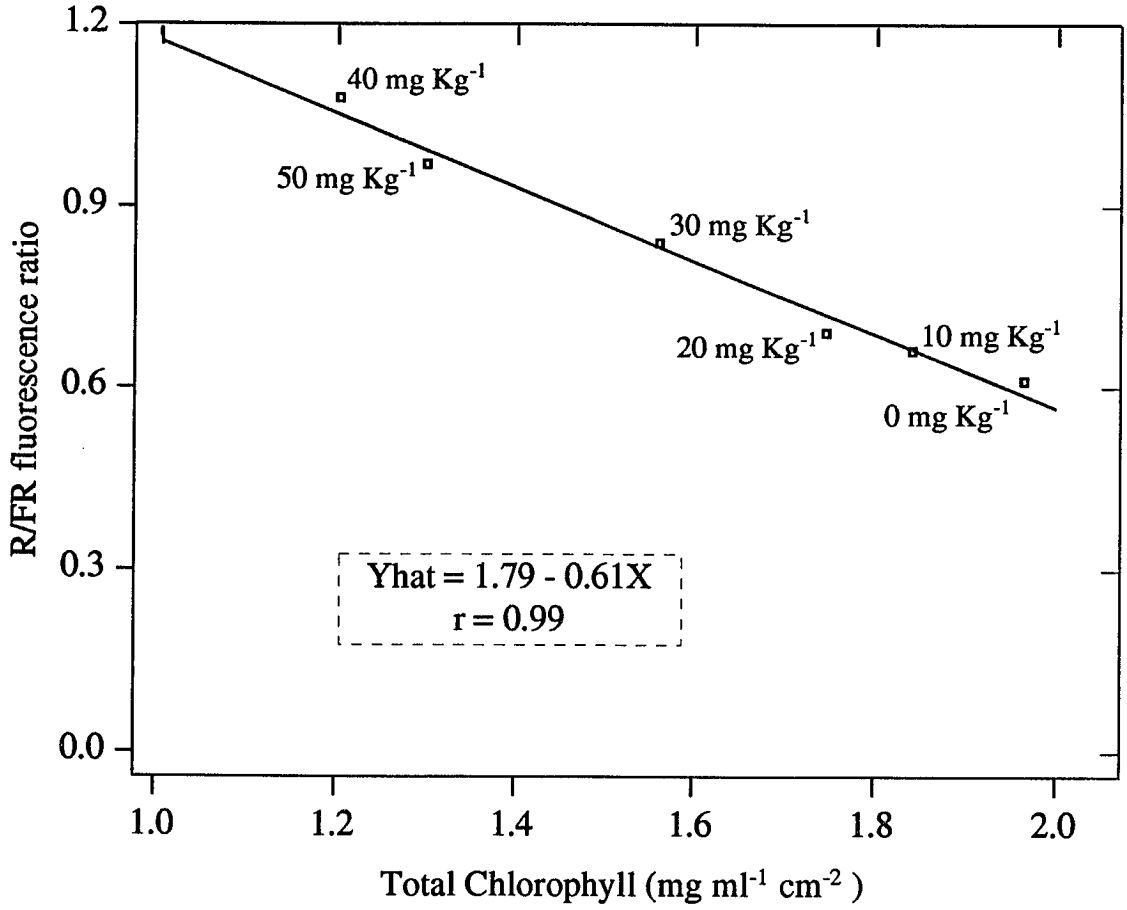

Figure 19. Chlorophyll vs mean R/FR fluorescence ratios for beans planted in April, 1994.

Correlations are shown with the least squares fit line (n = 10), and the data points are labeled with the cadmium dasage.

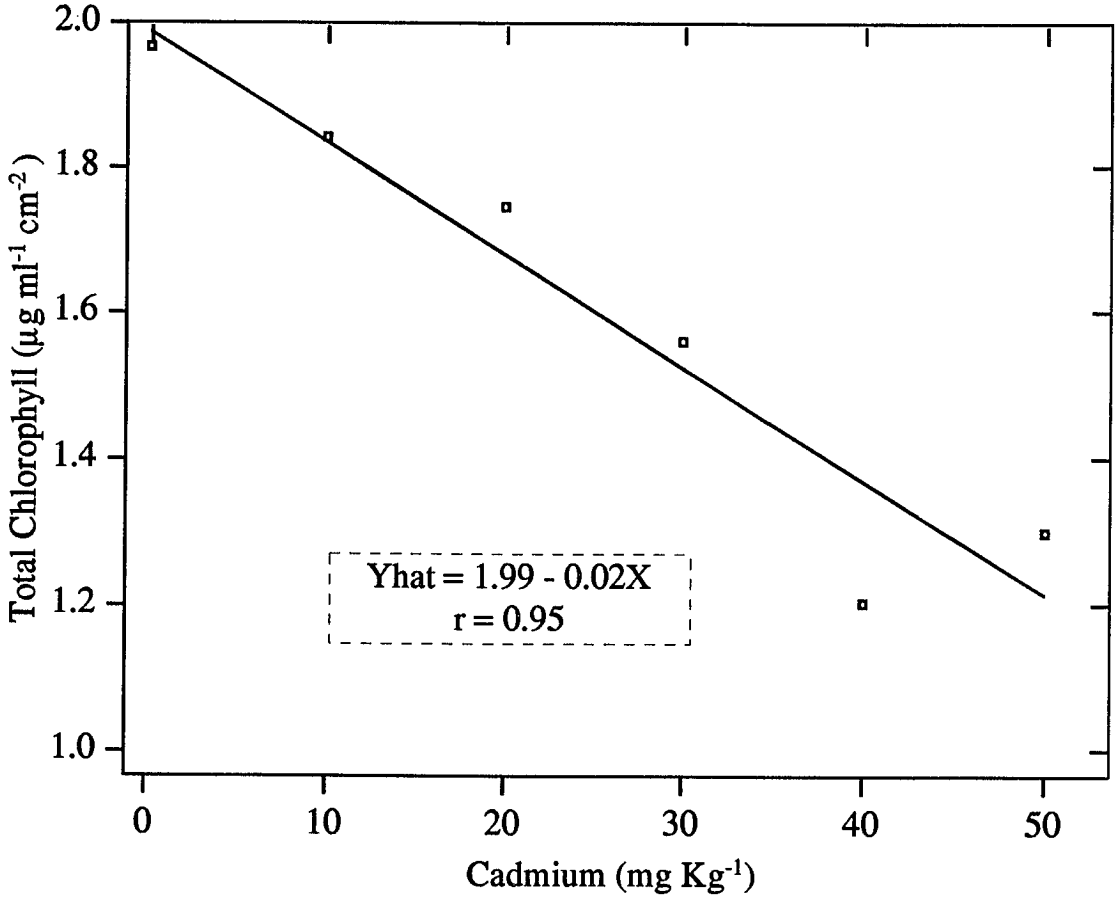

Figure 20. Cadmium vs chlorophyll for beans planted in April, 1994.

Correlations between the mean total chlorophyll values (n = 10) and cadmium dosage are shown with the least squares fit line.

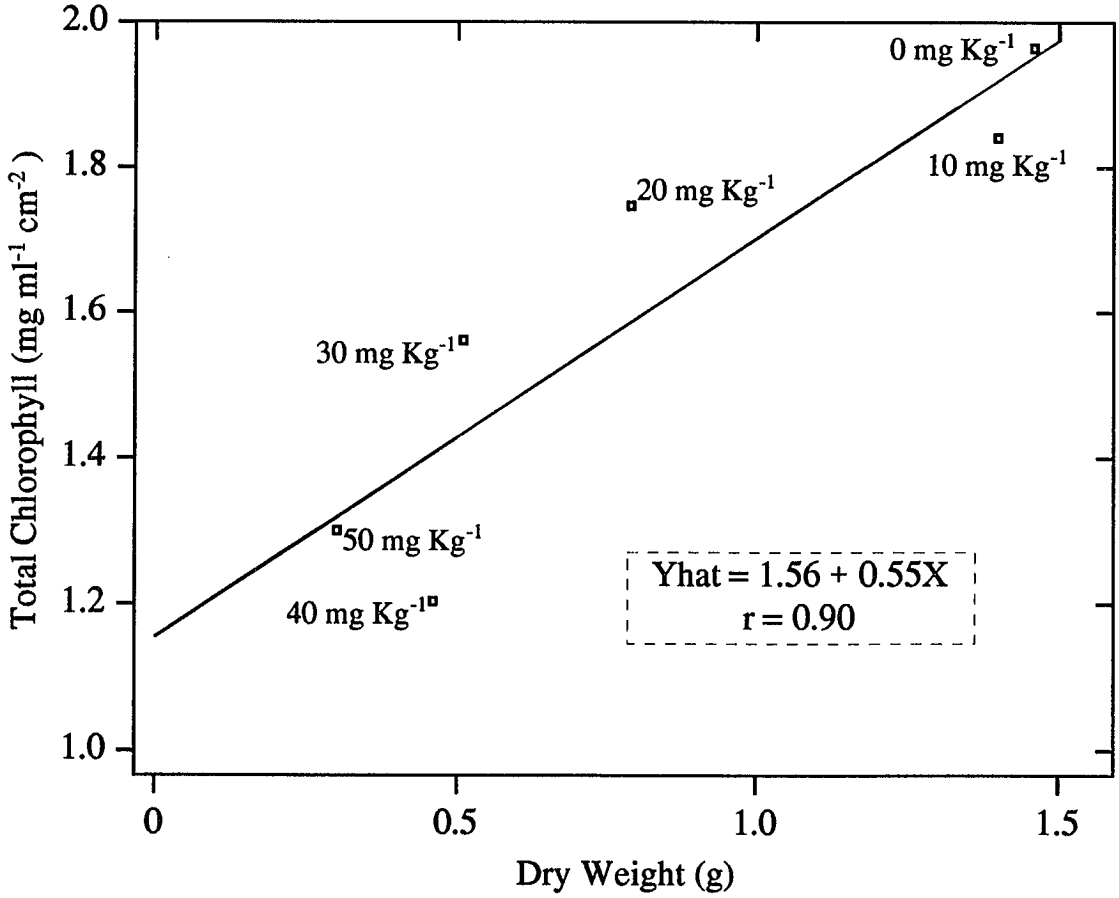

Figure 21. Dry weight vs chlorophyll for beans planted in April, 1994.

Correlations are shown with the least squares fit line (n = 10), and the data points are labeled with the cadmium dasage.

CHAPTER III[4]

NUMERICAL METHODS ANALYSIS OF EXCITATION-EMISSION-MATRIX FLUORESCENCE OF WATER STRESSED VEGETATION

ABSTRACT

Monitoring of water stress in vegetation is an important global economic and social function for remote sensing. Short-term water stress (dehydration) is often used in laboratory experiments to simulate long-term water stress (drought) due to time constraints. However, past investigations have noted differences in plant biochemical changes between drought and dehydration conditions. The biochemical changes occur in light collecting pigment-proteins with known absorption characteristics that can, in turn, affect fluorescence emission. Analysis of fluorescence emission spectra obtained with remote sensing instruments is becoming an important method for monitoring the physiological status of plants during water stress. Detecting changes in pigment-proteins *in vivo* would provide information for the refinement and improvement of existing fluorescence collecting techniques. We have found a high correlation between the 3rd derivative of excitation spectra and the in vivo absorption maxima known for the light harvesting complex components (chlorophylls *a* and *b*, and carotenoids). The calculation by numerical methods of the 3rd derivative of the excitation spectra from excitation-emission-matrix (EEM) fluorescence data from water stressed plants was used to determine differences in the light absorption by beans (*Phaseolus vulgaris* L.) subjected to drought and dehydration. Our results indicate that this technique is capable of determining the effects of the different water stressing conditions at the molecular level, and that future experiments, using instruments capable of greater spectral detail, can be applied to the task of determining appropriate wavelengths for remote sensing.

4/ This Chapter was submitted for publication to *Remote Sensing of Environment.*

INTRODUCTION

Famine and starvation are a constantly repeating occurrence for many parts of the world and desertification is a growing concern as forested areas are eliminated for fuel and the raising of crops and livestock. Water stress is ubiquitous in all but a few regions of the world (Aber and Melillo, 1991) , and drought limits plant growth and crop productivity more than any other single environmental factor (Sharp and Davies, 1989). While the effects of drought are most profoundly personal, the job of managing a solution is local or regional, and the consequences of failure to achieve a solution can be global.

Water stress detection in several species of plants has been a target for the use of remote sensing instruments as a means of monitoring vegetation health status in response to changes in water availability. A number of techniques have been developed for the analysis of the data obtained by various remote sensing instruments. The development of relative water content indices from remotely sensed reflectance data has been conducted by several investigators (Choudhury, 1987; Rouse et al., 1973; Rouse et al., 1974). These studies provided information on the health status of plants and were complimentary to other remote sensing methods that detected a blue shift of the red edge (Collins et al., 1983; Rock et al., 1988; Vogelmann et al., 1993), and reflectance changes in the shortwave infrared (Rock et al., 1986; Carter, 1993). However, recent papers question the use of reflectance data to monitor plant water stress. Cohen (1991) reports that as great a variation was found in the reflectance of a collection of leaves of the same species, all subjected to the same level of water stress, as was found for leaves, also of the same species, at differing levels of water stress. Cohen's conclusion was that the failure to extract treatment effects from the general population limits the applicability of reflectance remote sensing for plant canopy water stress monitoring. In airborne sensor studies of trees induced to water stress by severing the sapwood, Riggs and Running (1991) found small differences in Norway spruce and and no differences in white pine, between the reflectance data obtained for stressed and control plots of the two species.

Because of the extensive ground data required to interpret the differences that were seen in the airborne sensor data, they were lead to the conclusion that operational application of reflectance data to water stress detection, on the scale to which it is best suited, may not be feasible. It is apparent that alternative methods being pursued for the remote sensing of plant health status need to be examined more carefully. Several recent investigations have used laser instruments to analyze the fluorescence characteristics of vegetation, in various states of health, from a distance (Cecchi et al.; Camenen et al.; McMurtrey III et al.: all 1994). Passive techniques for the collection of vegetation fluorescence have also been demonstrated (McFarlane et al., 1980; Brach, 1983).

Dehydration is often used to simulate drought conditions in the laboratory because of the shorter time required to conduct such experiments. However, several investigators have shown that a difference in plant response exists between long-term water stress (drought) and short-term water stress (dehydration). Farquhar et al. (1989) note multiple and often contradictory reports of differing effects when various species of plants were subjected to drought or dehydration. Massacci and Jones (1990) state that drought had a marked effect on both gas exchange and chlorophyll fluorescence quenching as contrasted with dehydration which affected only gas exchange. Havaux et al. (1987) state that changes in photoacoustic signals were found to be similar for plants subjected to either drought or dehydration. They note however, that dehydration produced damage in the oxidizing side of photosystem II (PSII) whereas drought resulted in a strong decrease in photochemical energy storage and a lowering of the apparent absorption cross section of PSII with the reaction center itself as the primary site of damage. Dreyer et al. (1992) concluded that sensitivity of the photosynthetic apparatus to leaf dehydration in the absence of irradiance plays a minor role in the adaptation of plant species to drought, and that a decrease in photosynthetic activity in response to water stress under natural conditions is probably due mainly to stomatal closure along with high light and heat levels. Sharp and Davies (1989) point out that abscisic acid (ABA) can be released from

its normal site within the chloroplasts by increases in xylem sap pH, that ABA, synthesized in root tips in response to slight dehydration, can be transported to the leaves, and that stomatal closure can be initiated by increases in xylem sap ABA. Ogren (1990) suggests that dehydration is different from drought at the mechanistic level as judged by the fluorescence characteristics. Different excitation spectral changes are reported by Embry and Nothnagel (1994) for drought induced senescence than for the induced senescence of excised leaves. No remarkable increase occurs in the 400 to 500 nm wavelength range for drought stressed plants, but a slight increase occurs in the 450 to 520 nm range for the dehydrated leaves. They further note that a major reduction occurs in the excitation spectra from 430 to 500 nm late in the dehydration experiment. Their findings suggest that the carotenoid contribution to light harvesting energy collection does not increase relative to that of chlorophylls in drought induced senescence. Embry and Nothnagel (1994) state that, in the dehydration experiment, the initial increases reported for the 450 to 520 nm range of the excitation spectra occurred before a decline in the total chlorophyll to total carotenoid ratio proceeded very far. The diversity of responses to water stress may therefore reflect, in part, the diversity of signals from turgor, water content, and pH of leaf cells and xylem sap, to root response to soil strength and water content (Farquhar et al., 1989), and to the method of water stress (i.e. drought or dehydration).

The purpose of this study was to, 1) demonstrate the ability to use steady-state fluorescence to detect differences between dehydration and drought stressed beans (*Phaseolus vulgaris* L.) and, 2) confirm that quantitative information can be extracted from excitation-emission-matrix (EEM) data sets beyond the qualitative observations possible with the full visible and near visible excitation and emission changes presented at one time, and the usual removal of individual excitation or emission spectra and the data-point ratios derived from them.

EXPERIMENTAL METHODS

Fluorescence Measurements

Fluorescence response was measured with a Perkin-Elmer MPF-44B Fluorescence Spectrophotometer using techniques described below (reference to brand names is made as a matter of convenience and does not imply endorsement by the authors, the University of New Hampshire, or the sponsors of this research). The instrument has scanning monochromators for both excitation and emission, each with a measuring range from 200 to 900 nm. Both excitation and emission slit widths are adjustable for bandwidth limiting, and a ratio mode is used wherein the gain of the sample detector is controlled by a reference detector to minimize the effects of any potential drift in the excitation source lamp. The source lamp is a 150 watt Xenon powered by a current-stabilized supply. The signal detectors, sample and reference, are Hamamatsu R928 photomultipliers. A light excluding extension was added to the sample compartment of the laboratory fluorescence spectrophotometer to accommodate whole, live plants grown in pots up to six inches in diameter. After passing through the excitation monochromator, entrance slit and focussing lens, the 1 mm by 10 mm beam of light strikes the sample, set in a holder at 35° to the incident beam. This minimizes the possibility of reflected excitation light reaching the emission optics and detector.

For this study, emission intensity levels were collected at 5 nm increments, from 360 to 800 nm at excitation wavelength steps of 10 nm from 300 to 600 nm. This sequence of measurements required approximately 45 minutes to complete. A series of long-pass filters were used to minimize the effects of scattered light at emission wavelengths near the excitation wavelength, and also to eliminate second order harmonics of the excitation light that can otherwise be detected by the emission photomultiplier. The fluorescence intensity data thus collected constitute an EEM file.

Even with all parameters of the instrument in alignment, the collected spectra may not be considered "true" representations of the emission and excitation spectra of the

sample. The response characteristics of the instrument excitation source, photodetector, and excitation and emission optics, that vary with wavelength, need to be taken into account so that resulting emission spectra, and ratios derived from these spectra, can be confidently compared with like data collected with other fluorescence instruments. To correct for these variations, the response of rhodamine B dye, in high concentration such that it becomes a quantum counter, is compared to a constant output level over the excitation range to be used for data collection in order to provide an excitation correction file. An emission correction file is produced by comparing the light from a traceable-standard tungsten lamp to the calculated blackbody emission of tungsten for the same color temperature. The raw spectral data are corrected using the values in the correction files before the spectra are stored on floppy disk. These correction files are updated with a Xenon lamp change or detector realignment.

Vegetation

The experiments were carried out in the University of New Hampshire greenhouses. Bean plants were grown in 6 inch pots in neutral (pH 7) soil that contained 100% base saturation, very high levels of calcium (2663 mg Kg^{-1}), magnesium (287 mg Kg^{-1}), and phosphorus (40 mg Kg^{-1}), and low levels of potassium (125 mg Kg^{-1}). At the time of planting, 250 mg Kg^{-1} NPK (nitrogen:phosphorus:potassium) 20:20:20 was added to the pots. Lights were used to extend day length to 14 hours, and temperatures in the greenhouse were maintained near 20° C during the daytime and 10° C at night with heat pipes located beneath the benches. Humidity varied with outside ambient conditions. Watering was provided on an as needed basis by greenhouse staff who checked on the plants multiple times daily.

Fluorescence measurements were made on intact and attached leaves by choosing an interveinal location for the excitation beam to illuminate, and repeated measurements of the same leaf were made as near to the originally chosen spot as possible. The drought

stress portion of the experiment was begun 45 days after planting on 13 September 1993. The EEM fluorescence measurements were begun approximately one hour after the last time of watering, near mid-day, and continued daily at mid-day until the permanent wilting point was apparently reached 14 days after the initial measurements. At this time it was impossible to position the excitation beam at the originally chosen location due to the wilting. The dehydration portion of the experiment was then begun, 67 days after planting. Initial EEM measurements were made on a leaf still attached to the healthy, well watered plant. Excision of the leaf from the plant followed the initial measurements, and the leaf was left in the sample holder, in the fluorescence spectrophotometer until the apparent permanent wilting point was reached at 5 days following initial measurements.

Numerical Methods

The EEM data set provides multiple possibilities for the use of the information contained within. Emission spectra provide information about chlorophyll content and photosystem functionality (Lichtenthaler et al., 1986). The authors have used emission spectral data exclusively in previous reports (Theisen et al., 1994; Theisen and Rock, 1994) mostly because of the time constraints associated with the use of the EEM method (i.e. the 45 minute scan time), although some of the emission spectral data were extracted from EEM data sets. Excitation spectra, when they have been properly corrected, provide a near exact replica of the absorption spectra of higher plant pigments (Strasser and Butler, 1977a; Siefermann-Harms, 1987; Nothnagel, 1987), and can, in this context, be considered as the same. The excitation spectra of a whole, *in vivo* leaf are composed of individual spectra of the multiple pigments in a complex mixture that have been represented as gaussian curves for some analyses (Brown and French, 1959; Thornber et al., 1976). The formulas and coefficients of the actual spectra are unknown, and derivatives of unknown equations cannot be calculated. There are ways however, to use

the raw data directly to obtain the equivalent of derivatives. Al-Khafaji and Tooley (1986) note in their engineering textbook that a mathematical solution to a real world problem should be general, involve few simplifying assumptions, and be versatile in handling different boundary and initial conditions. This is the essence of numerical methods and they present the equations below for calculating approximations of derivatives from tabulated, equally spaced data from a series of differences. While the absorption of light by the several pigments of light harvesting complex aggregates within thylakoid membranes does not represent the exact same kind of problem as an engineer would encounter in building a bridge, the pigments are bound by the same laws of physics, and useful information is hidden in the raw data obtained from these structures that can be extracted with the same mathematical tools. The formulas for both forward and backward nth differences are combined to provide as close an approximation of the *n*th derivative as is possible with equally spaced tabulated data. The formulas are:

$$\Delta^3 f_i = f_{i+3} - 3f_{i+2} + 3f_{i+1} - f_i \text{ and} \quad (1)$$

$$V^3 f_i = f_i - 3f_{i-1} + 3f_{i-2} - f_{i-3} \quad (2)$$

where f_i is any given data point, and i+1, i+2, . . . i+n span equal intervals.

RESULTS AND DISCUSSION

Six of the EEM data sets for the drought stress portion of the experiment are displayed in 3-D perspective plots in Figures 22a through 22f. Because there was no significant difference in the measurements until day 11, only the EEM data for days 0, and 10 through 14 are shown. The changes due to dehydration occurred more rapidly, and the EEM data for the entire experiment time, days 0 through 5, are shown in Figures 23a through 23f. From a qualitative inspection of the EEM data for drought stress (figure 22) an increase can be seen in red emission (680 to 700 nm) relative to far-red emission (ca 740 nm) up to day 13, when a collapse in the blue-green region of excitation relative to

the red excitation region is evident, and inspection of the EEM data for dehydration stress (figure 23) shows an increase in red versus far-red emission up to day 4. Quantitative analysis of these EEM data sets provides the red/far-red (R/FR) fluorescence emission ratios, calculated at an excitation of 480 nm, and overall intensity maxima presented in Tables 3 and 4. In both cases there is a steady increase until the apparent permanent wilting point is reached followed by a drop in overall intensity in the final EEM plots (day 14 and 5, respectively). The increase in R/FR ratio is consistent with the findings of Epron and Dreyer (1992) who note a decrease in both photochemical and nonphotochemical fluorescence quenching when dehydration produced a water deficit above 0.3 megapascals (MPa) and a decrease in photosynthetic functionality with a water deficit above 0.75 MPa. The increase in R/FR is also consistent with a loss of chlorophyll for leaves suffering from a decrease in water potential (Alberte and Thornber, 1977) and the similar results produced by senescence (Lang and Lichtenthaler, 1991). The drop in overall intensity seen in the last EEM data set of both experiments may be explained by an increase in the loss of energy from the leaves as heat (radiationless dissipation, K_D) when the plant or leaf has reached a state of photoinhibition. Demmig-Adams et al. (1989) note that when a plant is exposed to water stress, photosynthetic activity decreases (i.e. with closed stomates, internal CO_2 is reduced and assimilation declines). With decreased CO_2 assimilation, normal light levels become excessive and K_D and zeaxanthin content rise. The xanthophyll zeaxanthin is a reversible reaction product of violaxanthin with the former more plentiful in chloroplast envelopes in the light and the latter more plentiful in the dark (Lawlor, 1990). It was presumed, though not yet demonstrated, that zeaxanthin differs from violaxanthin by more readily losing energy as heat (Farquhar et al., 1989). The differences in intensity between the blue-green and red excitation regions seen in the drought EEM of day 14 but not in the equivalent dehydration EEM data for day 4, can be explained by the fact that the blue-green region is dominated by pigments found only in the light harvesting complexes (Evans and Seemann, 1989). This may be an

indication that the reaction center pigments are being favored in preference to light harvesting pigments as put forth by Stuhlfauth et al. (1990) The differences in overall fluorescence intensity between the two experiments, EEM maxima and the R/FR fluorescence ratio magnitudes (shown in Tables 3 and 4), are most likely the result of the increased age of the bean plants at the start of the dehydration portion of the experiment.

Stuhlfauth et al. (1990) withheld water from *Digitalis lanata*, decreasing the water potential from -0.7 to -2.5 MPa, and they noted that the contents of chlorophylls *a* and *b* were not significantly changed, but that β-carotene increased by about 25% and the xanthophylls lutein, neoxanthin and violaxanthin decreased by 15 to 30%. In their study of the senescence of proso millet (*Panicum miliaceum*), Embry and Nothnagel (1994) induced senescence by withholding water from both intact plants and excised leaves. They observed small changes in the excitation spectra of the plants that they attributed mostly to a decrease in the chlorophyll *a*/*b* ratio. A decline in the total chlorophyll to total carotenoid ratio occurred earlier in the senescence process but these changes were not identified with excitation spectral changes by the authors. The excitation spectra presented showed maxima in the 400 to 500 nm region that Embry and Nothnagel indicated were the result of Soret bands of chlorophyll *a* and *b* and carotenoid pigments. Soret bands are produced when electrons are boosted to high excited states by the greater energy released by short wavelength photons. A maximum in the 600 to 700 nm region was stated to be solely due to the first excited states of chlorophyll *a* and *b*. No attempt was made to isolate the contributions of the various pigments.

The question of identifying the absorption maxima of higher plants has been investigated for a long time. French (1960) published an extensive tabulation of pigment absorption features, in ethyl ether, but also gave a comprehensive explanation of the difficulties involved in the measurement of plant pigment absorption *in vivo*. Sauer and Calvin (1962) analyzed spinach (*Spinacia oleracea* L,) quantasomes to ascertain the wavelengths of absorption maxima for the multiple pigments contained therein. They

compared their findings with other in vivo absorption measurements and concluded that the wavelengths represent the expected values for the common in vivo higher green plant. The *in vivo* absorption maxima from Sauer and Calvin that fall within the excitation range of our experiments are included in Table 5. Note that the wavelength values given in Table 5 should be accepted with the caveat that internal plant biochemistry can shift them towards the red or blue from a few to tens of nanometers (French, 1960). Sauer and Calvin (1962) identify the in vivo maxima for chlorophyll *a* and *b*, but the maxima for all carotenes and xanthophylls are combined. Ruban et al. (1993) isolated the absorption maxima of 6 xanthophylls in ethanol (ca 440 and 470 nm). Unfortunately for this study where a 10 nm excitation interval was used, the maxima of the xanthophylls differ by 10 nm or less. Carotenoids *in vivo* commonly exhibit absorption maxima shifts of about 10 nm towards longer wavelengths from those obtained in ethanol as a result of their occurrence in association with a lipid or protein environment, and the red shift is not the same for each carotenoid (Britton, 1983). The red shift of the carotenoids is consistent with that seen for chlorophylls (Katz et al., 1976).

To assist in the analysis of absorption spectra for the purposes of identifying absorption maxima, French (1960) used a technique of difference spectra. For the investigation of the yield of energy transfer and the spectral distribution of excitation energy, Strasser and Butler (1977b) also used difference spectra to identify absorption maxima, but included the use of the first derivative for further refinement of the maxima wavelengths. They determined that corrected excitation spectra provide a good match for absorption spectra. Thornber et al. (1976) interpreted several forms of chlorophyll a from the absorption spectra of the P700 complex of blue-green algae by using computer-assisted gaussian curve-fitting and the fourth derivative of low temperature absorption spectra. Purcell et al. (1985) made use of the fourth derivative to identify the individual chemical components from the synchronous emission scan of a mixture of pure phenols.

Figures 24 presents the excitation spectrum from 370 to 600 nm, collected at 10 nm intervals, of the 735 nm emission from the day 0 dehydration stress EEM data set and the calculated 3rd derivative approximation of that spectrum. The positive maxima at 380, 400, 420, 430, 450, 470, 480, and 590 nm are an extremely good match to the higher green plant pigment absorption maxima shown in Table 5. Maxima seen at 530 and 560 nm are not included in Table 5, but French (1960) shows minor absorption maxima near these wavelengths for chlorophylls *a* and *b* in ethyl ether, and Voet and Voet (1990) and Chappelle et al. (1992) both present figures of absorption spectra of the chlorophylls *a* and *b* that clearly show minor maxima near 530 and 560 nm. One maximum for chlorophyll *a*, listed in Table 5 (437 nm), unaccountably corresponds to a strong negative peak in the 3rd derivative of the excitation spectrum. The 3rd derivative approximation plot does not show an absorption or excitation spectrum, but rather the maxima associated with the known absorption wavelengths of the pigments identified in Table 5.

The 3rd derivative approximation was calculated for the excitation data of all the EEM data sets shown in figures 22 and 23 for the drought and dehydration stress experiments. The results are shown in contour plots in Figures 25a through 25f (drought) and 26a through 26f (dehydration). Only the positive contours (maxima) are shown for clarity. A lower signal to noise ratio, to which the 3rd derivative is sensitive, for the drought EEM data, produced contour plots that were more difficult to interpret. However, all the maxima were present, at least for the initial measurements, though not as clearly defined as those for the dehydration EEM data.

We investigated the points of potential spectral importance made by Stuhlfauth et al. (1990) and by Embry and Nothnagel (1994), as stated above. The lack of change in chlorophyll *a* and *b* content seen by Stuhlfauth et al. was apparently not repeated in our experiments due to the greater level of stress to which the plants and leaves were subjected. In our experiments there is a general rise in intensity of the fluorescence over time attributable to a gradual loss of CO_2 assimilation, a decrease in both photochemical

and non-photochemical fluorescence quenching, and a decrease in photosynthetic functionality that can be seen in the raw data maxima and R/FR fluorescence emission ratios, but not seen, or necessarily expected, in the 3rd derivative maxima.

Carotenoid aggregation (Ruban et al., 1993) may be the basis for the dramatic spectral shift towards the blue seen in the drought 3rd derivative plots beginning on day 12 and peaking on day 13. Whereas a prominent maxima is found at 480 nm with a subsidiary maxima at 470 nm for all the dehydration 3rd derivative plots and for the drought 3rd derivative plots from day 0 though day 11, the major peak by day 12 is no longer either 480 or 470 nm but 460 nm. Ruban et al. (1993) attribute absorption spectral shifts towards either the red or blue to different types of aggregation of carotenoids, and suggest that these spectral changes are related to non-photochemical quenching *in vivo*. While other evidence in the data presented above suggests the occurrence of non-photochemical quenching in the final stages of both the drought and dehydration experiments, the spectral shift seen in the 3rd derivative of the data for the drought stressed beans is opposite in nature to that reported by Ruban et al. for the non-photochemical quenching effect that they relate to the xanthophyll cycle. Ruban et al. point out that the xanthophylls aggregate to different degrees, with zeaxanthin having the strongest tendency for aggregation. The shift towards the blue may therefore be related to the differential tendency towards aggregation and the increase of β-carotene and decrease in xanthophyll seen by Stuhlfauth et al. (1990).

CONCLUSIONS

Steady-state fluorescence presented in the form of EEM 3-D perspective and 3rd derivative contour plots has been shown to be useful in detecting differences, consistent with the findings of previous researchers, between drought and dehydration stressed beans (*Phaseolus vulgaris* L.). Numerical methods and other forms of quantitative

analysis, applied to the EEM data sets can be used to extract important information. The 3rd derivative maxima at 380, 400, 420,430, 450, 470, 480, and 590 match the previously confirmed *in vivo* maxima of chloroplast pigments chlorophyll *a* (383, 418,, and 594 nm), chlorophyll *b* (470 nm), and carotenoids (400, 428, 455, and 485 nm), all occurring in bean leaves. While there is a lack of a matching 3rd derivative positive peak for the chlorophyll *a* absorption maximum at 437 nm, an explanation for the blue shift from 480 nm to shorter wavelengths in the drought stress experiment, that is not readily apparent in other forms of data presentation, is suggested by the work of previous investigators. Our results indicate the viability of a technique that can be used in future experiments with instruments with the capability of collecting EEM data more rapidly and with greater spectral definition (i.e. with smaller excitation intervals). Such experiments, supported by a better understanding of the *in vivo* wavelengths of pigment absorption, especially among the carotenoids, can only lead to a better understanding of the *in vivo* response of plants to stressing conditions that are essential to the development of effective remote sensing tools.

TABLES

Table 3. Overall intensity maxima and red/far-red (R/FR) fluorescence ratios for drought stressed beans.

Day	R/FR	maxima
0	0.31	10.17
10	0.31	8.40
11	0.37	8.98
12	0.62	9.80
13	0.76	19.91
14	0.54	9.21

Table 4. Overall intensity maxima and red/far-red (R/FR) fluorescence ratios for dehydration stressed beans.

Day	R/FR	maxima
0	0.52	94.45
1	0.59	242.22
2	0.71	253.09
3	0.81	272.67
4	0.90	289.63
5	0.80	121.50

Table 5. Pigment absorption wavelengths from spinach quantasomes (Sauer and Calvin, 1962) representing *in vivo* absorption maxima for higher green plants.

Pigment	wavelength	nearest 10 nm (relative to EEM excitation)
chlorophyll *a*	383	380
"	418	420
"	437	440
"	594	590
chlorophyll *b*	470	470
Carotenoids	400	400
"	428	430
"	455	450
"	485	480

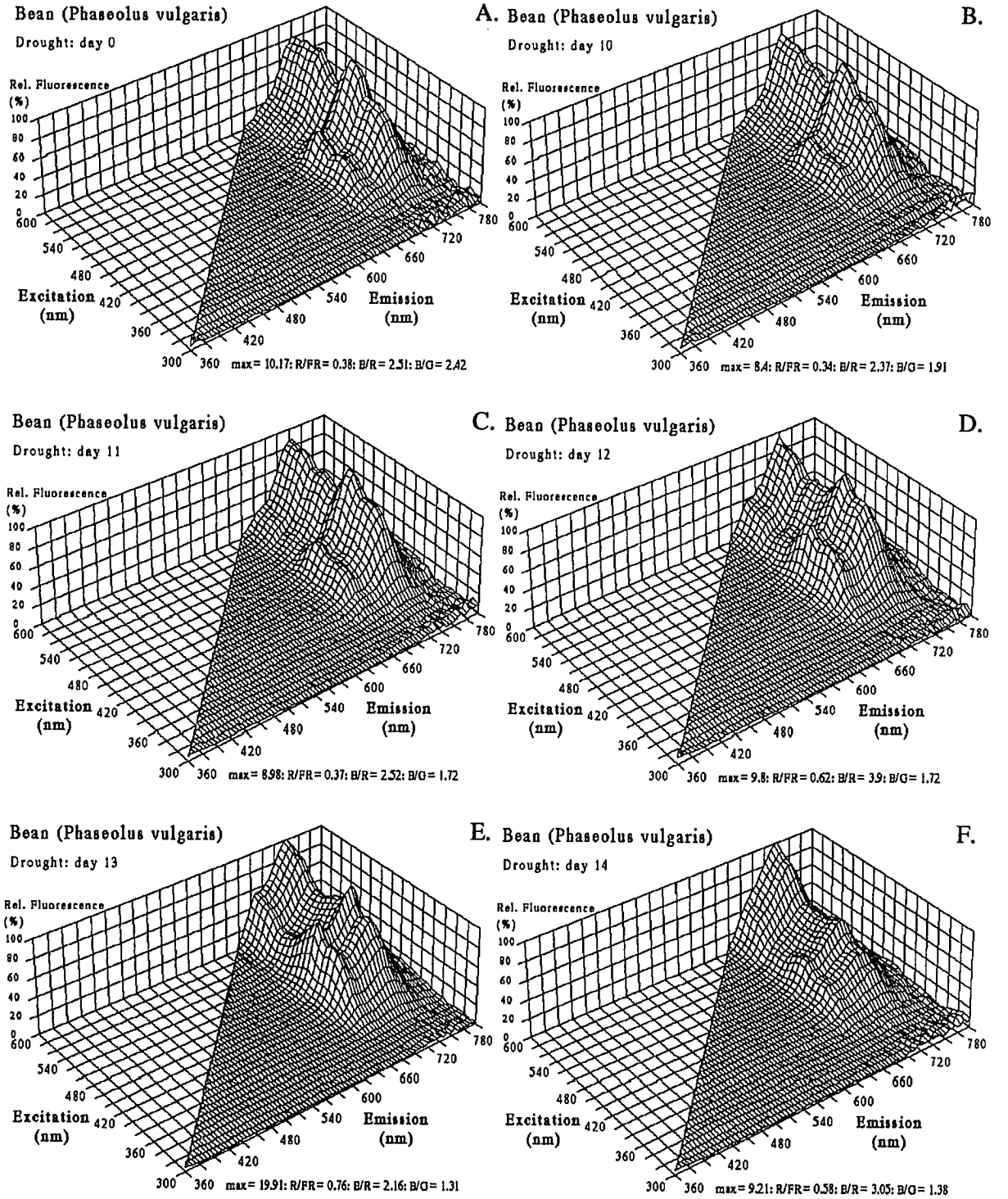

Figure 22. EEM data from drought stressed beans.

Data is normalized to the maxima for each data set for days 0, 10, 11, 12, 13 , and 14 (a though f, respectively).

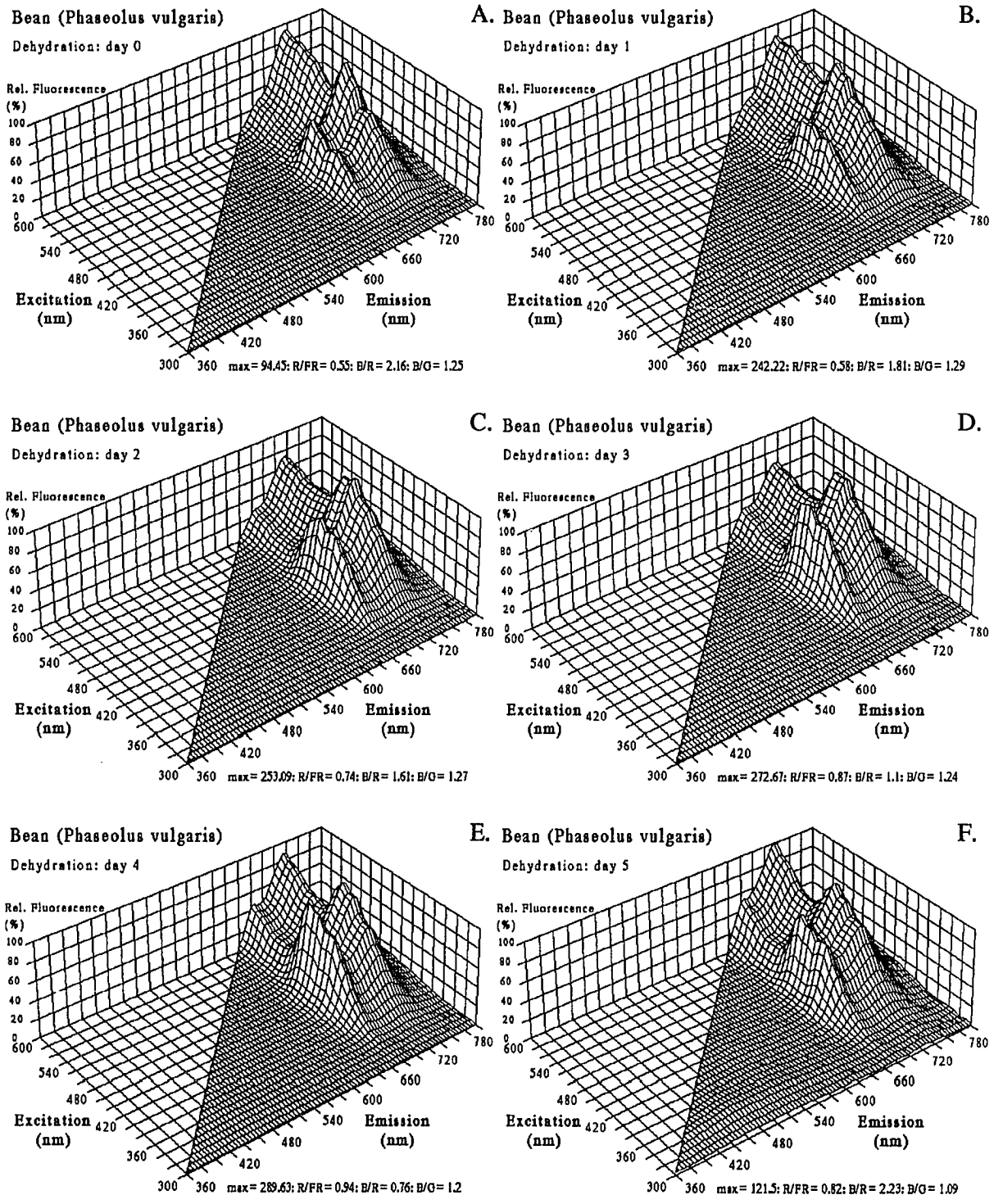

Figure 23. EEM data from dehydration stressed beans.

Data are normalized to the maxima for each data set for days 0 through 5 (a though f, respectively).

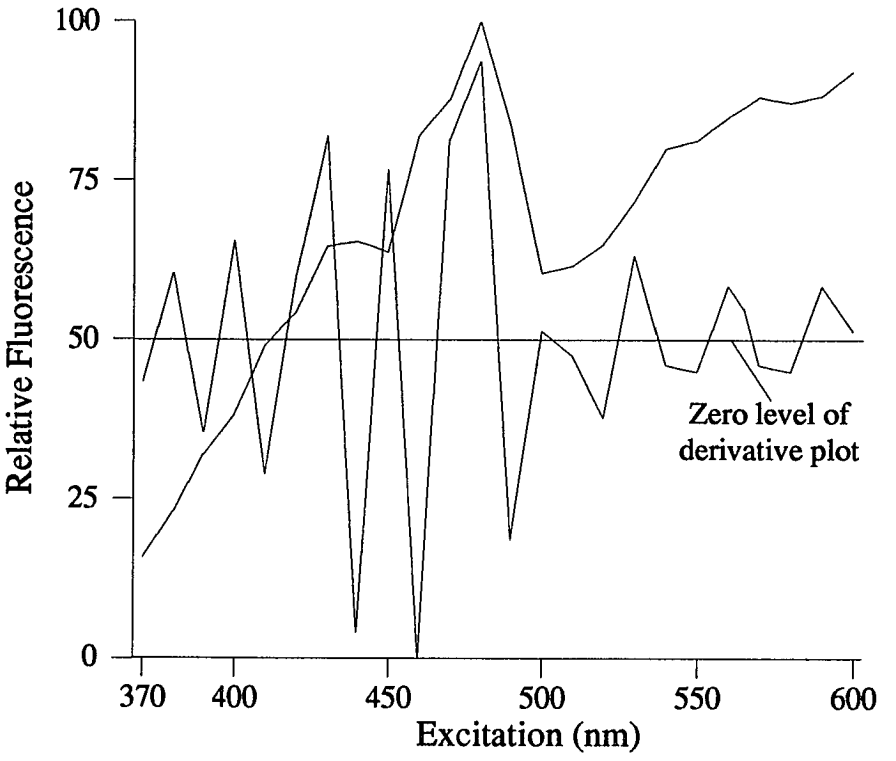

Figure 24. Excitation spectrum and 3rd derivative.

Dehydration stress day 0 normalized raw excitation spectrum from 370 to 600 nm with the 3rd derivative.

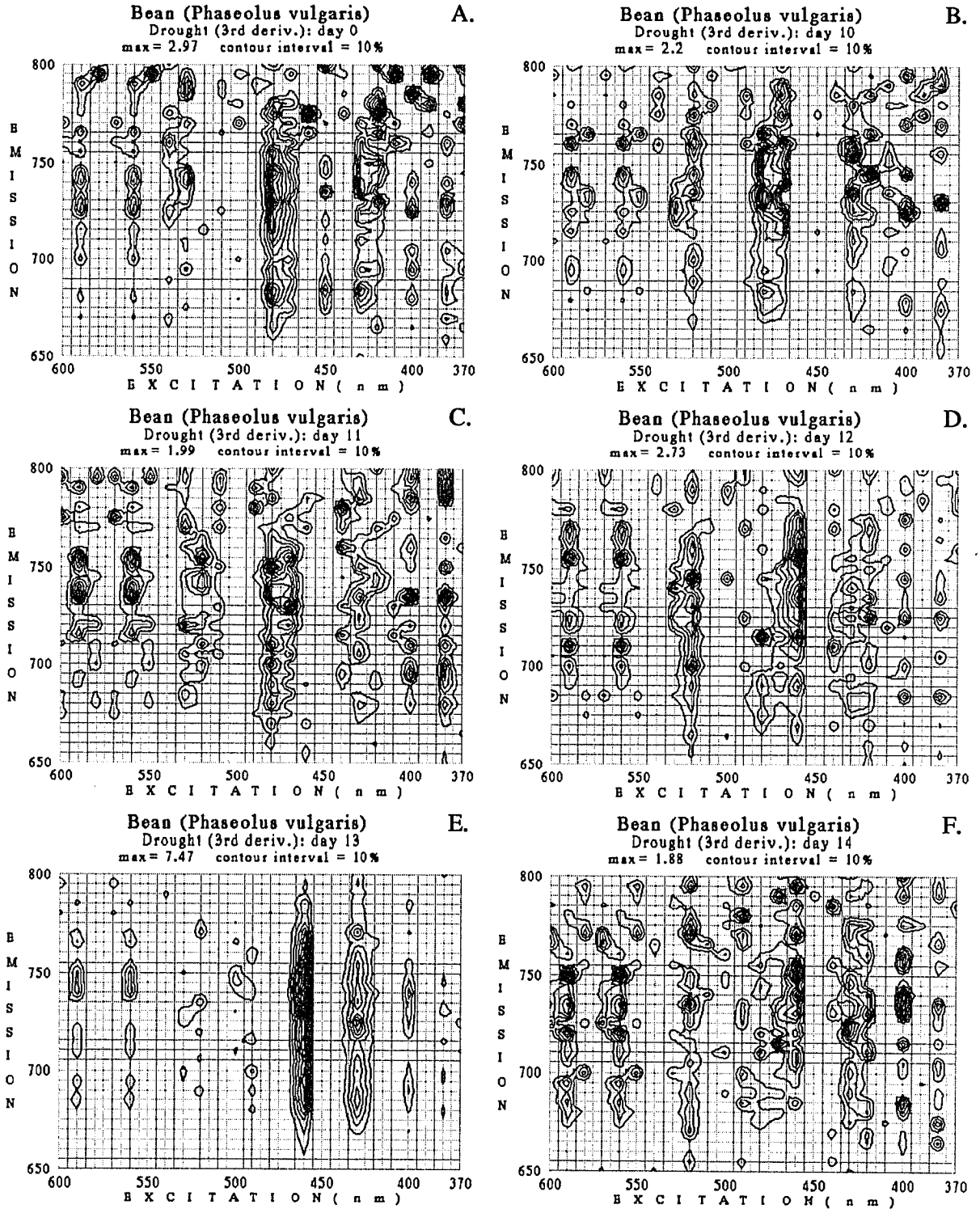

Figure 25. Contour plots of the excitation 3rd derivative.

The drought stress EEM data sets for days 0, 10, 11, 12, 13 , and 14 (a though f, respectively) are shown.

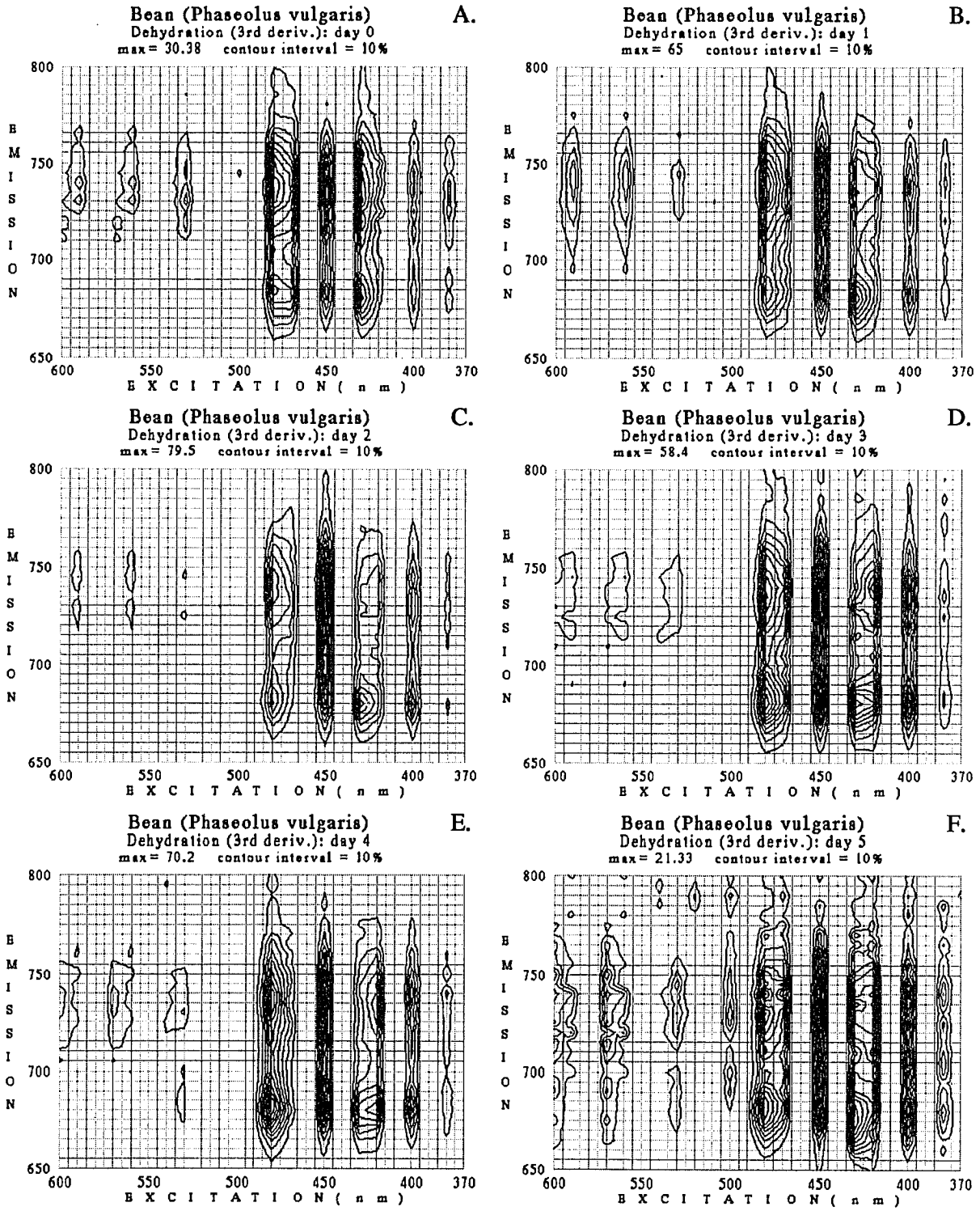

Figure 26. Contour plots of the excitation 3rd derivative.

The dehydration stress EEM data sets for days 0 through 5 (a though f, respectively) are shown.

CHAPTER IV[5]

AUTOMATING A LABORATORY INSTRUMENT FOR CHLOROPHYLL FLUORESCENCE RESEARCH

ABSTRACT

Older laboratory instruments need not be counted out simply because they were not designed with the latest computer controls. For those institutions and investigators whose financial resources are limited, there are alternatives. This paper describes the relatively simple modifications that were made to a fluorescence spectrophotometer so that an inexpensive personal computer could be used to increase its capability and the productivity of the project.

INTRODUCTION

Tight budgets in research and development, especially at smaller universities or in small government projects, have been a fact of life for many of us during most of our careers. More investigators will be confronted with this situation during the 1990's as every type of business and institution tries to make the most of limited assets. The analytical instrument companies, to their great credit, have continued to upgrade the capabilities of their products while keeping prices stable. As examples, and without intension to favor any one particular company, Hitachi of America and Shimadzu recently announced their newest fluorescence spectrophotometers, capable of collecting data 60 times faster than the instrument currently used by this author. The cost of these new instruments was not significantly different from that paid for a very similar, but now older instrument purchased in 1980. Even so, acquiring the latest laboratory instruments,

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5/ This Chapter was submitted for publication to *American Laboratory.*

at least obtaining the use of the newest equipment, may not be possible. Others may have to make do with what they have, and the intent of this paper is to share the results of our efforts in this regard.

In a previous paper for *American Laboratory*, Theisen and Hemphill (1985) described how a non-automated laboratory fluorescence spectrophotometer (Perkin-Elmer MPF-44B) could be coupled with a personal computer so that data could be collected rapidly and efficiently. That modification was incomplete, as it allowed the computer to collect and store data, but still required the operator to manually set and reset the monochromators after each scan, open and close the excitation source shutter, and start and stop the monochromator drive motors. Zung et al. (1988) described a method for using a personal computer to control a stepper motor equipped fluorometer (Perkin-Elmer 650-10S) with the same goal in mind. Upon reading the Zung paper, the first thought was to replace the monochromator drive motors in the MPF-44B with stepper motors. However, an alternate solution was chosen that did not require replacement of the motors or the acquisition of additional controlling hardware.

The requirement for faster and more efficient data acquisition, in 1985, was based upon the desire to produce excitation-emission-matrix (EEM) fluorescence information for rocks and minerals similar to the EEM's described for chemical solutions by Warner et al. (1977). The EEM fluorescence information made available with the enhanced instrument has been used to explore important questions in mineralogy (Hemphill et al., 1988; Tyson et al., 1988). Later in 1988, the EEM method was applied to the study of plants (Theisen, 1988).

THE MODIFICATION

Plants, because they are living entities, unlike rocks and minerals of course, have dynamic fluorescence characteristics. The emissions associated with plants change

quickly in response to environmental conditions. As a consequence, when an experiment is designed to test a species of plant's reaction to a given stimulus, and measure the fluorescence changes associated with the plant's reaction, many plants must be used for a given treatment to achieve statistically significant results.

To allow for the measurement of whole, living, potted plants, while maintaining the light-excluding integrity of the MPF-44B fluorescence spectrophotometer sample compartment, an extension was added. The advantage of this modification is that individual plants, leaves or needles can be measured repeatedly during the growing season. This capability, an advantage of fiber-optic or telescopic spectrometers, is combined with the, usually greater, spectral and data storage capabilities of the laboratory spectrophotometer. The photograph (figure 27) shows the extension that was added to facilitate the measurement of whole, live plants in pots up to 6 inches in diameter.

Some of the original electrical and electronic modifications did not have to be altered. The voltage associated with emission intensity and voltages indicating switch closures for hardware-determined emission and excitation wavelength steps, and the start of the monochromator drive motors fed to an Apple IIe microcomputer via a Cyborg/ISAAC hardware/software laboratory interface, remain as described in the earlier paper (Theisen and Hemphill, 1985). However, rather that use only the input capability of the ISAAC, relays were wired in parallel with the drive on/off switches for both emission and excitation, the monochromator drive direction control switch, and the excitation source shutter closure switch, and these relays were connected to the digital-to-analog (D/A) outputs of the ISAAC (figure28).

The fourth relay (figure 29), used to control the excitation-source shutter, is an on/off switch wired in parallel with the front panel shutter open/close switch. Also shown in figure 29 is the manner in which all the relays are controlled by the ISAAC D/A outputs.

All of the electronic parts were available at an electronics supply outlet, such as

Radio Shack. Wiring connections and transistors were soldered directly to relay socket pins and insulated standoffs after the sockets and standoffs were mounted on an aluminum plate through appropriately sized holes. A 9-pin computer connector was used to join the relay wiring to the ISAAC.

APPLICATIONS

Software to collect fluorescence data in various formats was modified to make use of the new control features. The operator is still required to make the initial excitation and emission wavelength settings, monitor the initial photomultiplier (PMT) voltage, set the monochromator scanning speeds, and load the appropriate order-separating filters into their holders. Instructions to remind the operator of all manual functions are displayed on the computer monitor.

For EEM data collection, the program expects the excitation and emission shutters to be open. The program then closes the excitation shutter, pauses, "reads" a dark level, opens the excitation shutter, pauses again to allow the PMT intensity to stabilize, and starts the emission scan. The program "reads" the wavelength step switch voltage, and then "reads" the PMT intensity at 5 nm intervals until the operator-selected ending wavelength is reached. At the end of an emission spectral scan, the program closes the excitation shutter, reverses the monochromator drive motor, and starts the drive motor. The wavelength step switch is "read", as the monochromator is driven in reverse, to determine the correct stopping point. If filter changes are required the operator is informed. When the change is completed the program repeats the process until all the emission spectra for the desired excitation wavelengths are acquired. At the end of EEM data collection, the data is stored on floppy disk, the emission and excitation monochromators are reset to the starting wavelengths, and the excitation shutter is opened. Power is applied to the shutter relay to close the shutter; therefore, to save wear

on this device, manual control of the front panel shutter switch is used between data collections.

EEM data sets for plants (figures 30a and 30b) are composed of an excitation range from 300 to 600 nm in 10 nm steps. Emission spectra are collected from 60 nm beyond the excitation wavelength to 800 nm for each excitation wavelength. Each emission data point intensity value, at 5 nm intervals, is the average of 15 readings at a specific wavelength. This procedure is used to limit the effects of high frequency instrument noise related to the use of non-cooled photomultipliers.

The emission spectra collected at the shorter excitation wavelengths (below 400 nm) provide information on "blue" and "green" fluorescence (Virgin, 1956; Chappelle et al., 1984; Chappelle et al., 1990; Lichtenthaler and Strober, 1990). The emission spectra at two longer excitation wavelengths (470 and 600 nm) provide excitation-emission peak ratios for analysis of carotenoid/chlorophyll electron transport (E470/E600 at 685 and 735 nm emission, Gruszecki et al., 1991) and red/far-red (R/FR) ratios for the analysis of photosystem II / photosystem I electron transport or total chlorophyll levels (i.e. the F685/F735 ratio at 470 nm excitation).

The above information, and more, can be extracted from EEM fluorescence data arrays. Note the information below the plots in figure 30. Either excitation or emission spectra for any wavelength, within the covered ranges, can also be extracted for separate illustration (see figure 31). However, with the currently available instrument, the advantage provided by the increased flexibility for data analysis inherent in the EEM arrays is outweighed by the increased time required to collect the full range of EEM data which, in turn, decreases the total number of samples that can be observed in a limited time period. An abbreviated version of the EEM data collection program, especially suited to the fluorescence of plants, was written to speed the gathering of predetermined subsets of emission spectra so that larger numbers of plants could be evaluated in a limited amount of time. This program was found to be extremely useful for a study of

eastern white pine subjected to short-term exposures of ozone (Theisen et al., 1994). With the latest modification, the limiting factor for the number of possible measurements of plants became the time required to place the plants in the sample compartment and remove them.

SUMMARY

With computer-control of the spectrophotometer, other programming options are apparent that would have been difficult manually. Programs have now been written to collect time-sequence emission spectra for drought-stress or herbicide response experiments. If an inexpensive way could be found to change the multiple filters required for near-UV to near-IR EEM data, time-sequence EEM fluorescence measurements could also be accomplished.

The simplicity of this modification should allow for the adaptation of the techniques described here to be applied by almost anyone with knowledge the electrical, electronic, and mechanical functions of an instrument that they have authority to modify. While there is still hope that funds will become available to purchase the "ultimate analytical tool", current research requires features of the fluorescence spectrophotometer, such as multiple filter changes, that have not yet been automated, even on the newest instruments. Understandably, the addition of such features to commonly available instruments would not be required by high volume analytical laboratories and would necessitate an expense that would not be acceptable to them.

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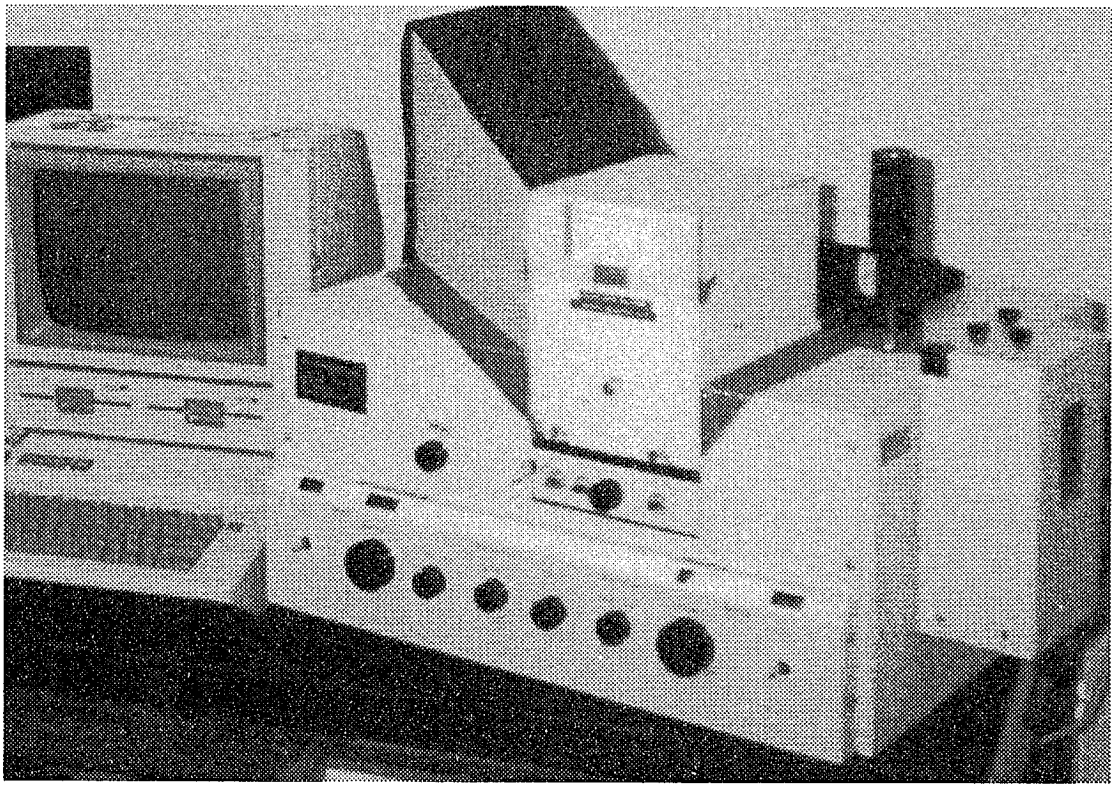

Figure 27. Modified fluorescence spectrophotometer, Perkin-Elmer MPF-44B.

An extension has been added above the sample compartment to maintain the light-excluding integrity while accepting live potted plants.

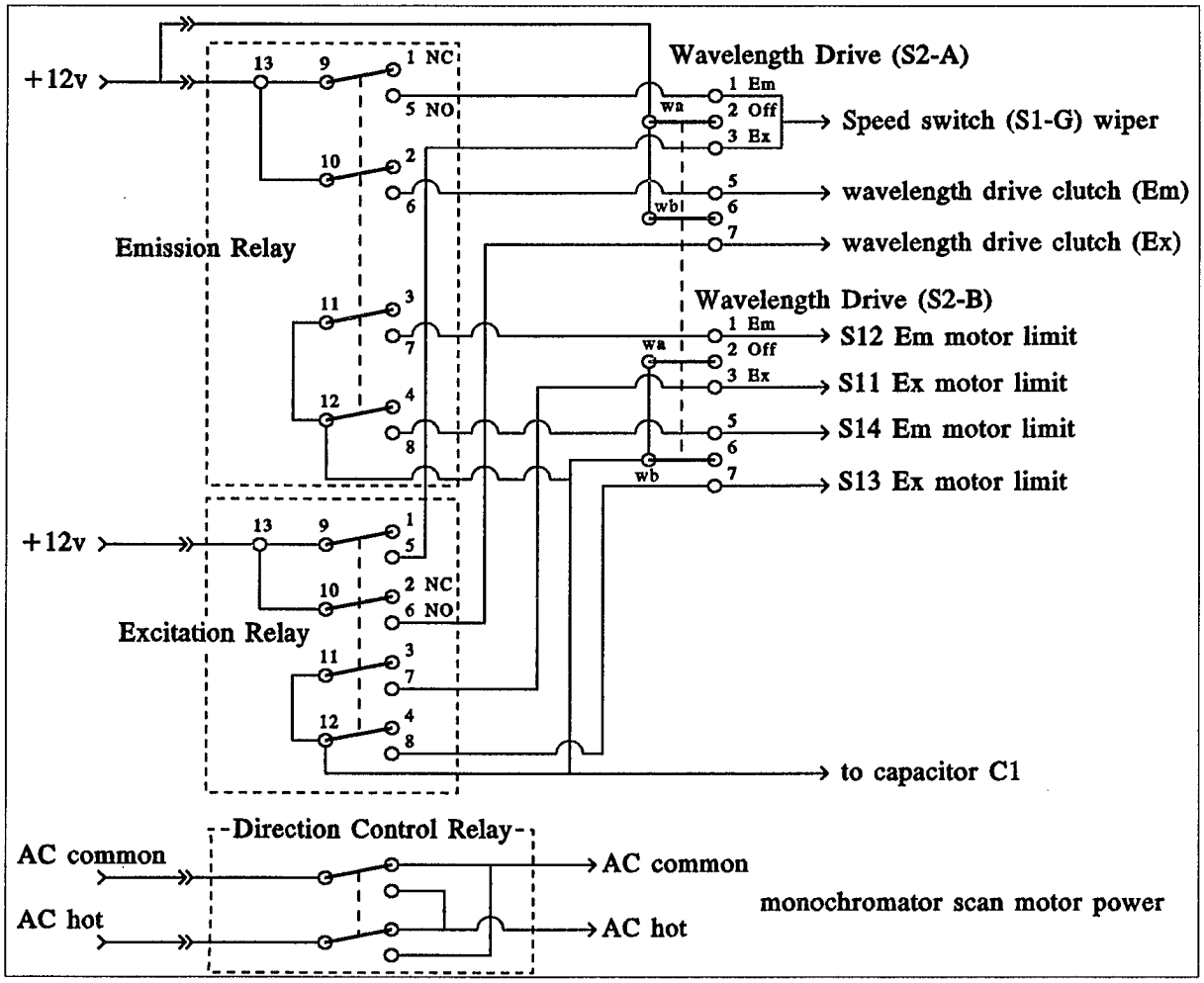

Figure 28. Schematic of the multi-contact relays for modification of the MPF-44B.

The schematic above shows how three of the multiple-contact relays are connected to the wiring of the Main Unit of the MPF-44B. Abbreviations are NC (normally closed), NO (normally open), and S2 etc. refer to switches in the MPF-44B Main Unit.

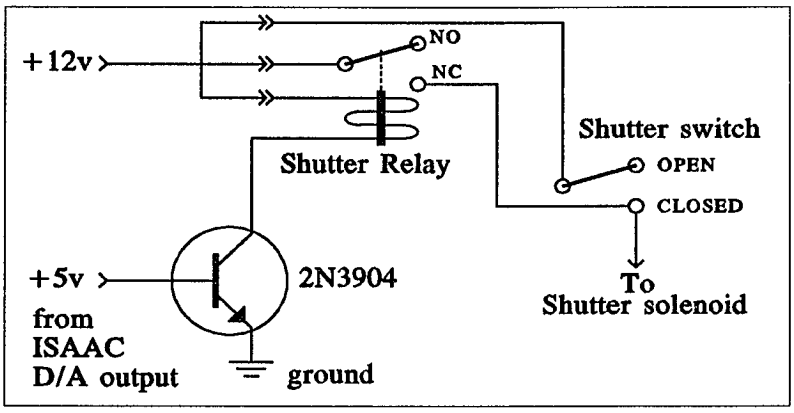

Figure 29. The excitation source shutter relay and switch connection schematic.

The schematic shows how a 5 volt signal sent from an ISAAC D/A output controls the relay through an NPN transistor.

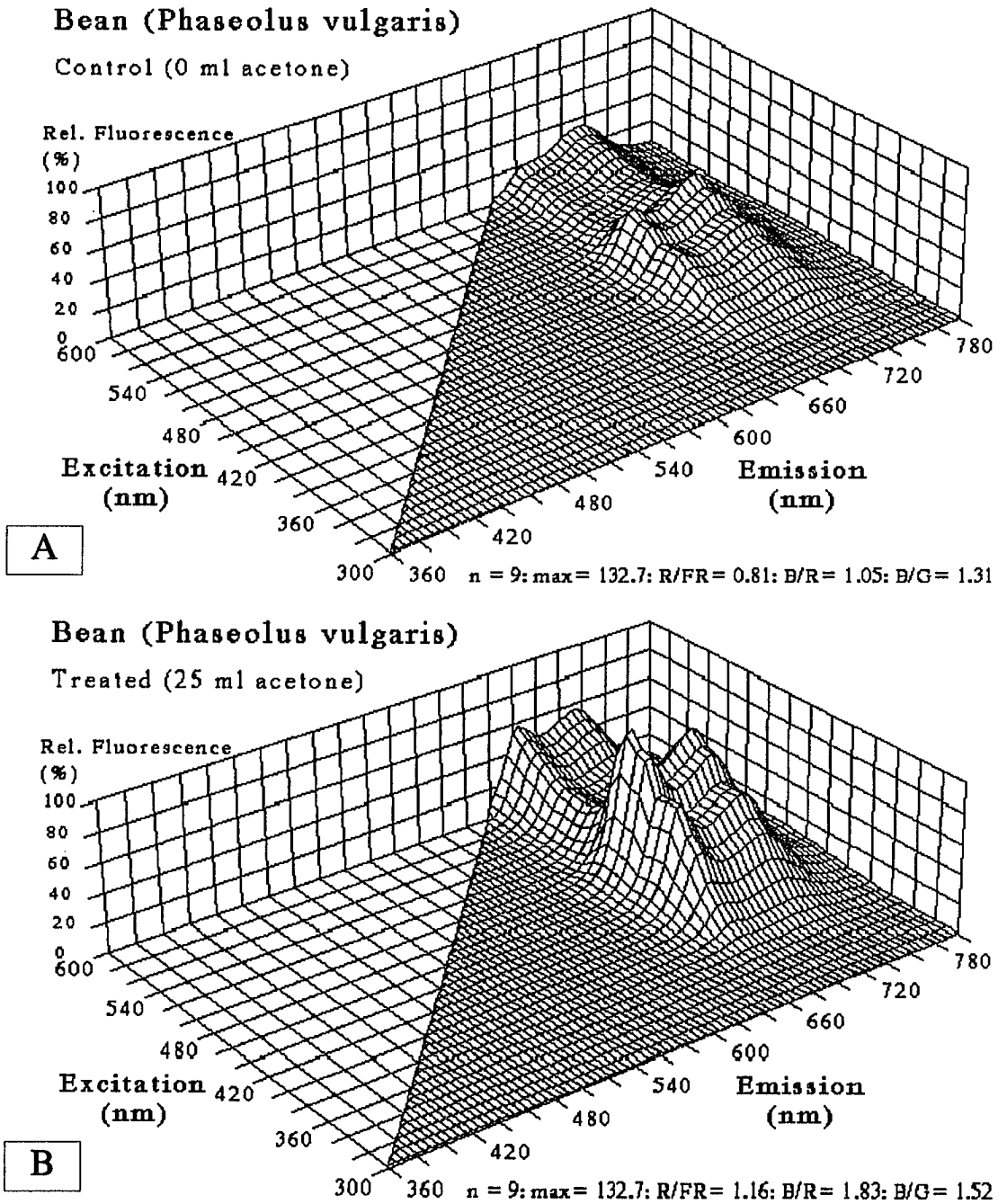

Figure 30. Excitation-emission-matrix (EEM) fluorescence data for bean plants.

The EEM data are presented in 3-D perspective plots representing the mean data of 9 EEMs each. Plot A shows data from untreated (Control) beans and plot B is data from beans treated with 25 ml of acetone after 15 days of growth. The fluorescence intensity for each plot was normalized to the maximum intensity for plot B. The fluorescence measurements were made when the plants were 49 days old.

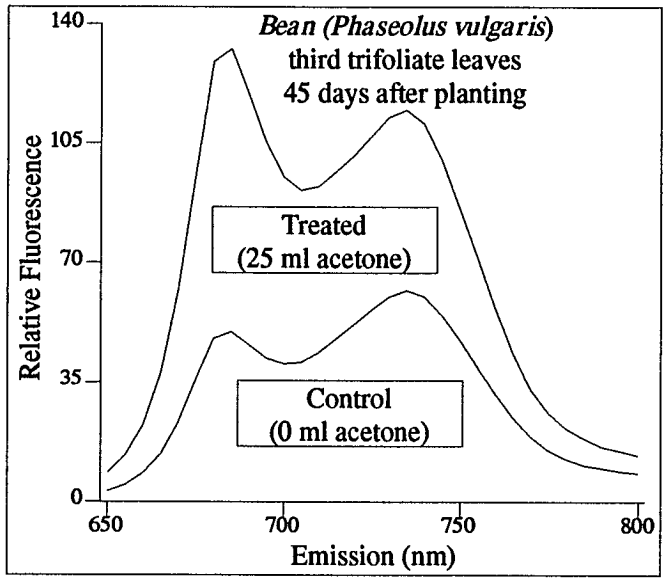

Figure 31. The mean fluorescence emission spectra of treated and control beans.

Treated and control beans are shown. The excitation wavelength is 480 nm; n = 9. Both red/far-red ratios and mean maxima show significant difference (P = 0.05) between the the two treatments. This relationship is as expected when one set of plants is subjected to conditions of stress (Lichtenthaler and Rinderle, 1988).

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