Laser Induced Fluorescence (LIF) as a Remote Sensing Tool
A REVIEW

[1]E.W. Chappelle, [1]M.S. Kim, [2]C.L. Mulchi,[3]C.S.T. Daughtry, [3]J. McMurtrey, and [1]L. Corp

Biospheric Sciences Branch[1]
Laboratory for Terrestrial Physics
NASA/Goddard Space Flight Center
Greenbelt, MD 20771
emmett@ltpmail.gsfc.nasa.gov

Department of Natural Resource Sciences[3]
University of Maryland
College Park, MD 20770

Remote Sensing and Modeling Laboratories[2]
ARS, United States Department of Agriculture
Beltsville, MD 20705

1. Introduction

Vegetational changes are primary indicators of the present and future ecological status of the globe. These are changes which not only impact upon the primary productivity, but the total of the biogeochemical processes occurring on the planet. The impacts of global climatic and other environmental changes on vegetation must be monitored by some means in order to develop models which will allow us to predict long term effects. Large scale monitoring is now possible only with remote sensing systems, primarily passive reflectance, obtained by the use of satellite and aircraft platforms. However, passive reflectance techniques at this time are limited in their ability to detect subtle changes in the concentration and oxidation states of the many compounds involved in the light reactions of photosynthesis. Knowledge of these changes we consider to be fundamental in the remote assessment of both the rate and efficiency of photosynthesis and also the early detection of stress damage. The above factors pointed to the desirability of a sensing technique with the sensitivity and specificity necessary for detecting and quantifying those biological entities involved in photosynthesis. Another optical technique for vegetation monitoring is fluorescence. Previously, the lack of adequate excitation light sources and detector technologies have limited the use of fluorescence on intact plant leaves in the field. It is only recently with the advent of lasers with short pulse duration and advanced detector technologies that fluorescence measurements in the remote

mode have become possible in the presence of ambient light. The use of steady state fluorescence techniques have been demonstrated to be feasible from *in situ* ground-based and airborne platforms (Kim, 1973; Hoge et al., 1983; Chappelle et al., 1984a, 1984b, 1985; Cecchi et al., 1998; Albers et al., 1995). Note that, since lasers are used as the excitation light sources to induced fluorescence, this active remote sensing method has become known as the laser induced fluorescence (LIF) techniques. The LIF spectra were found to be sensitive to changes in environmental parameters that are expected to change at the global-level. Our group has been conducting investigations into the use of LIF of vegetation for the assessment of certain vegetation parameters including primarily the integrity of photosynthesis and the effects of environmental stresses.

2. Background

Fluorescence is the phenomenon in which light absorption at a given wavelength by chromophore is followed by the emission of light, in general, at longer wavelengths (Stoke's shift). It has advantages over other spectroscopic methods mainly due to its high sensitivity. The distribution of wavelength-dependent intensity that causes fluorescence is known as the excitation spectrum, and the distribution of wavelength-dependent emission intensity caused by a given wavelength excitation is known as the emission spectrum. The steady-state fluorescence measurements have proven to be a versatile tool for a number of applications including cell biology, photochemistry, and environmental science.

Briefly, when molecules absorb photons in their ground state, they are elevated to the lowest excited state (or to one of the higher excited states). Energy in excess of its lowest excited state is rapidly dissipated into heat (internal conversion: IC). Upon returning to the ground state, part of energy is dissipated in the form of fluorescence by emission of a quantum of light at wavelengths corresponding to those of the lowest energy state (Chappelle and Williams, 1987).

Initially, the use of fluorescence measurements on higher plants (and algae) was confined to elucidate the fundamental mechanisms of photosynthesis. Kautsky and a co-worker in the early 1930s were the first to begin extensive investigations into transient characteristics of Chl fluorescence from dark-adapted photosynthetic materials, thus it is known as "Kautsky effect". Since then, chlorophyll (Chl) fluorescence as an analytical tool, studies have progressed to further enhance elucidation of the fundamental photochemistry of the photosynthetic apparatus in higher plants (and photosynthetic bacteria) using either *in vivo* leaves, or tissue homogenate or isolated chloroplast (Kok, 1965; Lavorel, 1977; Lictenthaler and Rinderle, 1988).

In the mid 1980s, Chappelle and his colleagues (1984a, 1984b, 1985) observed an additional broad fluorescence emission in the blue (F440) and green (F525) regions of the spectrum on intact plant leaves under UV-A (337 nm) excitation with maxima centered at 440 nm and an emission shoulder at 525 nm, respectively. They were the first to extensively use these additional fluorescence emission bands along with Chl fluorescence at the red (F685) and far-red (F730) regions of the spectrum with emission maxima at 685 and 730 nm as a remote means; for the assessment of plant vigor; and to differentiate major plant types (e.g., monocot, dicot, coniferous, deciduous). Since then, further studies have followed to illustrate the versatility of multispectral steady-state fluorescence emission characteristics from intact leaves in these regions of the spectrum (Chappelle and Williams,

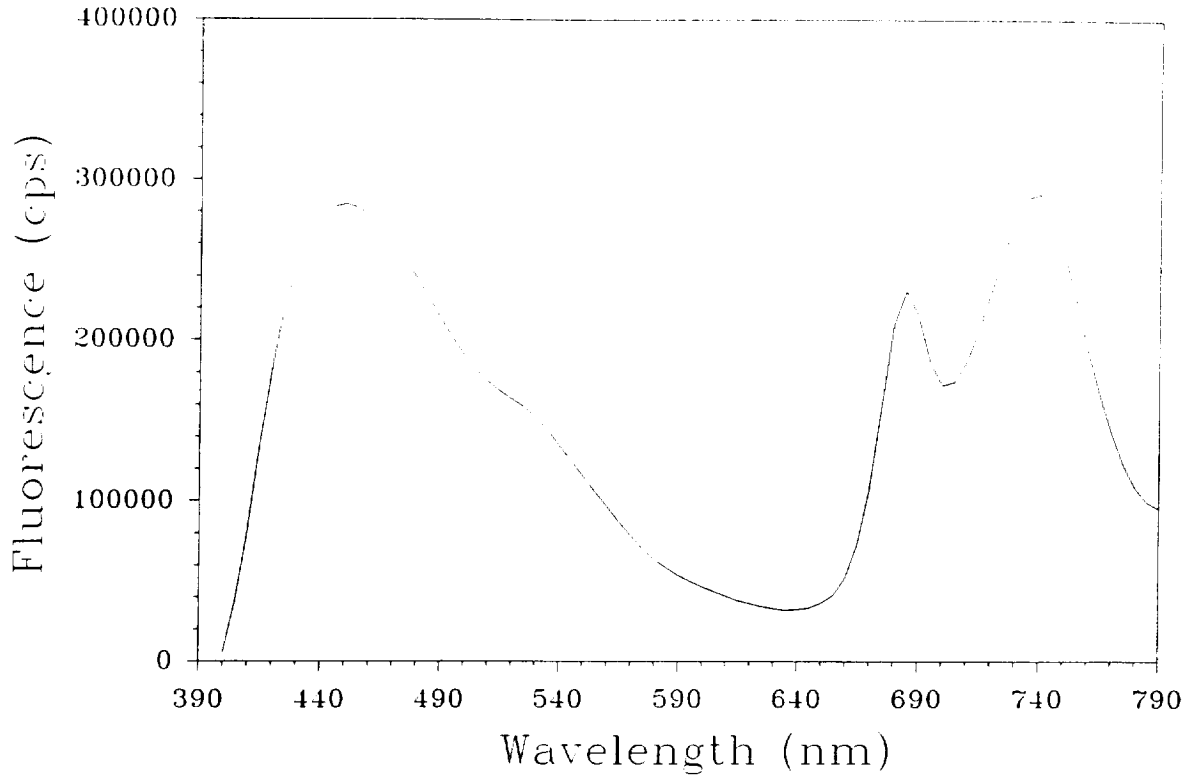

Figure 1 Fluorescence emission spectrum of a soybean leaf excited with UV radiation at 340 nm.

1987; Lang et al., 1992, 1996; Lictenthaler et al., 1990, 1996; Kim et al., 1998; Cecchi et al., 1998).

An example of the multispectral fluorescence emission spectrum of a normal soybean leaf with UV excitation (340 nm) at room temperature is illustrated in figure 1. It should be noted that the convoluted emission maximum at 525 nm region is not always distinctly seen under certain conditions and with certain plant species. This is not to say the compound responsible for F525 is absent, but its chemical state is such that fluorescence is not seen. The unit of the fluorescence intensity is an arbitrary unit and is expressed as relative fluorescence intensity (RFI). The appropriate excitation wavelengths for the blue-green fluorescence emission from intact leaves have been determined to be in the wavelength range from 310 to 400 nm (Chappelle et al., 1984, 1987; Schweiger et al., 1996). In addition, these excitation wavelengths yielded a significant Chl fluorescence emission at F685 and F730; however, the fluorescence yield increased toward the longer UV wavelength excitation. The magnitudes of the fluorescence bands have been found to change under several stress conditions and/or under growth environmental changes including both natural and those of anthropogenic origins; thus, these variations in spectral attributes may be utilized as a remote means to assess physiological state of plants (e.g., Chappelle and Williams, 1987).

2.1 Red (F685) and Far-Red (F735) Fluorescence

Because of the wide use of fluorescence techniques for fundamental photosynthesis studies, a wealth of information on the characteristics of Chl fluorescence bands is available. In a multi-pigment system such as photosynthetic apparatus, energy transfer is the first process that occurs after light absorption. Resonance energy transfer is the mechanism by which light energy absorbed by Chl *a* and the accessory pigments over the visible region of the spectrum are transferred to PS II Chl *a* in photosynthetic process. This is due to fluorescence of pigment species in close proximity to other pigment species which absorb light at wavelengths within the fluorescence emission band. Consequently, the energy transferred to photosystem (PS) II, but not utilized in photosyntheis, is dissipated as fluorescence at 685 and 735 nm regions of the spectrum, and as heat (Lictenthaler and Rinderle, 1988; Horton et al., 1993). Therefore, in general, an inverse relationship exists between Chl fluorescence and photosynthesis provided that the Chl concentration remains constant.

Although steady-state Chl fluorescence are usually measured with LIF, the transient fluorescence characteristics of Chl should be mentioned. Chlorophyll fluorescence at F685, when a dark-adapted leaf is suddenly exposed to a constant light, passes through a series of maxima and minima (Kautsky effect) before reaching a light adapted steady-state (F_s) level (Lictenthaler and Renderle, 1988). These transient fluorescence changes referred to as induction kinetics reflect the competition for the excited energy in PS II between fluorescence, photochemical quenching (qP), and nonphotochemical quenching (qN) where excited energy is dissipated as heat (Horton et al., 1994; Mohammad et al., 1995). The changes in qP reflects the photochemical redox potential in between PS II and PS I. Therefore, the magnitudes of induction kinetics may be subjective to the length and the history of the dark adaptation as well as light intensity.

Several studies have demonstrated that the ratio of the two steady-state Chl fluorescence peaks, namely F685/F735 can be used to assess relative Chl concentrations and photosynthetic efficiency (Hak et al., 1990; Lichtenthaler et al., 1993, 1996; Rosema et al., 1992; Stober et al., 1992, 1993a; Szabo et al., 1992; Agati et al., 1995). These observations were based on two key premises. First, F685 undergoes partial reabsorption by itself because of a strong absorbance of Chl *a* in the red region of the spectrum (Hak et al., 1990; D'Ambrosio et al., 1992; Hagg et al., 1992; Lictenthaler and Rinderle, 1988; Stober et al., 1992). Therefore, the F685/F735 ratio strongly depends on the Chl *a* concentration and decreases for plants with higher Chl *a* concentrations as reabsorption by Chl *a* at 685 nm become greater in comparison to that of 735 nm.

Second, the F685/F735 ratio was also shown to be sensitive to the photosynthetic activity of plant leaves. In plants treated with DCMU, a higher F685/F735 ratio was observed with respect to that of control leaves (Lictenthaler and Rinderle, 1988). These observations indicated the dependency of the ratio to photosynthetic electron transfer efficiency from PS II to PS I. Environmental perturbations that affect the photosynthetic activity of plants may be detected by this ratio. However, Chl fluorescence changes occur when stress conditions damage the photosynthetic apparatus as well as during temporary photoinhibition serving as a protective or adaptive mechanism.

Studies by Hak et al. (1990) and Stober and Lictenthaler (1992) showed decreased F685/F730 during the greening of etiolated leaves. D'Ambrosio et al. (1992) studied the F685/F730 ratio and Chl contents on nine tree species during the autumnal breakdown of Chl and showed increasing F685/F730 as leaves become senescent. They stated, thus the F685/F735 ratio may be used as a remote indicator of Chl content. The data presented by these investigations showed that the ratio and

Chl contents had an inverse-exponential (curvilinear) relationship which reached a plateau when the total leaf Chl contents reached approximately 15 to 20 $\mu g/cm^2$. These observations imply that the ratio may be a valid indicator of Chl contents up to only a certain concentration range. For instance, those conditions that resulted in significant variations in photosynthetic pigment contents on plants include nutrient deficiencies such as N (Heisel et al., 1996; McMurtrey et al., 1994).

In general, the Chl fluorescence ratio, F685/F730, increases synergistically in responses to both plant stresses that exacerbate the photosynthetic mechanisms and/or result in decreased Chl concentrations (e.g., either breakdown or less synthesis). The occurrence of F685/F730 characteristics suggested the potential exploitation of fluorescence techniques in the detection of physiological changes in plants induced by a number of abiotic and biotic factors which affect plant physiology. However, it was recognized that Chl fluorescence measurement alone may limit the use in relating such measurements to specific stress factors responsible for physiological changes in plants (Chappelle and Williams, 1987; Banninger et al., 1990). This may be more evident with such stress conditions accompanied by insignificant effects on the photosynthetic activities and/or those stresses causing insignificant changes (i.e., pre-visual) in photosynthetic pigments contents. There are additional fluorescence emissions in the blue-green region of the spectrum from intact plants when excited with UV radiation.

2.2 Blue (F440) and Green (F525) Fluorescence

Plant responses to environmental changes are complex interactions of biochemical as well as biophysical mechanisms which may result in subtle changes in concentrations and redox state of constituents other than Chl molecules. These may be compounds found in plants that may also fluoresce in the blue-green region of the spectrum under proper wavelength excitation (Chappelle et al., 1991). This also suggest that the compounds responsible for the blue-green fluorescence bands may be present in concentrations and oxidation states specific to a particular plant type due to genetic differences. Chappelle and Williams (1987) indicated that blue-green fluorescence may provide the additional information along with red and far-red fluorescence for the development of unique algorithms needed for the remote correlation of fluorescence measurements with plant stress conditions as well as species type differentiation.

The use of the blue-green fluorescence in vegetative monitoring is relative new with respect to Chl fluorescence. Uncertainties exist in terms of the identify of major constituents along with the precise location within the leaf, as well as other contributing factors/mechanisms governing the blue-green fluorescence emission characteristics from intact leaves (e.g., Goulas et al., 1990; Chappelle et al., 1987, 1991; Lang et al., 1991; Stober et al., 1993b; Lictenthaler et al., 1996). It is difficult to characterize factors responsible for blue-green fluorescence of *in vivo* plant leaves when considering the heterogeneity due to complex morphology and a web of biochemical/biophysical processes involved. Several investigations have been attempted to characterize the compounds and contributing factors responsible for the blue-green fluorescence from intact leaves.

It has been known that a number of compounds present in plants, such as polyphenolics, are fluorescent under UV excitation. Harris and Hartley (1976), based on a UV fluorescence microscopy study, illustrated that cell walls were highly fluorescent and suggested cell wall bound phenolics such ferulic acids as blue flourescent compound. Goulas et al. (1990) also suggested that free and esterified ferulic acids and *p*-coumaric acid as sources for blue fluorescence. Chappelle and Williams

(1987) speculated that *in vivo* fluorescence in the blue band of the spectrum may emanate from NADPH, tannic acid, lignin, vitamin K_1, and plastoquinone. Moreover, flavins and β-carotene were suggested to be responsible for green fluorescence emission. Morales et al. (1994) also showed that flavins such as FAD and FMN were responsible for *in vivo* green band emission.

In order to further assist in the identification of the compounds responsible for blue-green fluorescence emissions, fluorescence spectra of pure plant constituents as well as solvent extract were acquired (e.g., Chappelle and Williams 1987; Chappelle et al., 1991; Lang et al., 1991). Lang et al. (1991) suggested cell wall bound phenolics and compounds in vacuole such as chlorogenic acid, caffeic acid, sinapic acid, coumarins (aesculetin, scopoletin), and catechin, and stilbenes (*t*-stilbene, rhaponticin) as possible candidates for blue fluorescence. They also suggested alkaloid berberine and quercetin as compounds responsible for *in vivo* green fluorescence. Lang et al. (1991) believe that riboflavin may contribute little to green fluorescence.

Plant cell structural compounds such as lignin and phenolics are relatively inert chemically (Chappelle et al., 1991). The changes in the magnitude of blue-green fluorescence in responses to certain environmental stress conditions (e.g., Chappelle et al., 1984b, 1987) indicated that the fluorescence changes may be due to chemically dynamic (e.g., redox changes due to stress condition) molecules that may be involved in photosynthesis. Chappelle et al. (1991) showed that the blue fluorescent nature of water soluble extract from clover leaves in which was identified as due to NADPH that plays a vital role in P_n as one of the primary intermediates during the photosynthetic process, accepting electrons from PS I. They further showed that fluorescence ratio F440/F600 of greenhouse grown soybean leaves showed a strong positive linear relationship with the rate of photosynthesis.

Cerovic et al. (1993) showed that approximately 20% of the mesophyll blue fluorescence emanated from components located in the chloroplast of spinach (*Spinacia oleracea* L., var. Wobli). In addition, a light-induced change in blue fluorescence in the chloroplast and mesophyll cells were observed. This fluorescence change was suggested to be due to the reduction of NADP in PS I (Cerovic 1993). Above observations suggested that blue fluorescence emission changes may be more closely related to P_n than was previously thought. However, Stober and Lictenthaler (1993a) reported that no light-induced changes in blue-green fluorescence were observed during the induction kinetics of dark adapted soybean and wheat leaves.

Although the above compounds are considered to be responsible for the blue-green fluorescence emission from intact leaves, many investigations have suggested that photosynthetic pigments in the mesophyll layer which absorb strongly in these regions of the spectrum may selectively reabsorb the blue-green fluorescence emitted by other compounds (Lang et al., 1992; Stober and Lictenthaler, 1992; Stober et al., 1993b, 1994). This was based on observing a decrease in the blue-green fluorescence emissions during the greening of etiolated wheat leaves. Stober et al. (1992) reported an increasing blue to green ratio (F440/F530) as etiolated leaves turned green. They stated that blue fluorescence was reabsorbed by all the photosynthetic pigments and the green band was only partially reabsorbed by the accessory pigments. Morales et al. (1994) observed an increased blue-green fluorescence intensity in mesophylls of iron deficient sugar beet (*Beta vulgaris* L.) leaves. The iron deficiency treatments decreased photosynthetic pigments per unit area and they suggested that the most of the blue-green fluorescence increase was due to a reduction of the screening of UV light by photosynthetic pigments. In addition, selective reabsorption of blue fluorescence by Chl

molecules lead to changes in the form of the emission spectra. They suggested that the ratio decrease was due to accumulations of flavins in mesophyll as the result of iron deficiency.

Lang et al. (1991) observed significantly higher fluorescence intensities from the upper sides in comparison to the lower sides of bifacial C3 soybean leaves throughout the blue, green, red, and far-red regions of the spectrum. The contrast was speculated to be due to anatomical differences which resulted in a differential reabsorption by photosynthetic pigments. In a subsequent study by Lang et al. (1992) showed significantly higher green fluorescence intensities from the epidermal layer removed tobacco leaves than those of intact leaves, and thus they postulated that green fluorescence emanated solely from the mesophyll layers. On the contrary, since no significant blue fluorescence changes were observed between the intact leaves and the leaves stripped of the epidermal layers, blue fluorescence was suggested to emanate from cell wall in both the epidermal and the mesophyll layers. Moreover, because of the strong absorption and reabsorption by photosynthetic pigments in the blue region of the spectrum in the mesophyll layer, the majority of the blue fluorescence has been thought to emanated from the epidermal layers of intact green leaves. For instance, Stober et al. (1994) showed four fold higher blue fluorescence from wheat leaves treated with a bleaching herbicide norfluorazone (removal of photosynthetic pigments) compared to normal green wheat leaves. On the basis of this observation, they suggested that the major part of the blue fluorescence emanated from the cell walls of the epidermal layer of intact plants.

Several studies also indicated that UV screening and non-blue fluorescent compounds such as flavonols in the epidermis may attenuate the fraction of excitation beam reaching the mesophyll layer of the intact plants (Stober and Lictenthaler, 1993b; Stober et al., 1994; Lang et al., 1996). Stober et al. (1994) attempted to show this by removing photosynthetic pigments as well as UV light absorbing cell wall compounds, and the cuticle (e.g., cutin, lipids, and waxes) from wheat leaves with acetone. A ten-fold intensity increase at F440 were observed with respect to control leaves. Thus, they concluded that the absence of UV screening substances in the epidermis in conjunction with enhanced scattering characteristics due to the pigment free environment caused the increase in the blue fluorescence intensity. Cerovic et al. (1993) indicated that the epidermis in the intact leaves may absorb up to 75% of the UV exciting light before reaching mesophyll layer based on a comparison of F680 intensities between intact leaves and leave with the epidermal layers removed. However, their method to determine the UV attenuation may raise a concern since ruptured chloroplast cells may yield higher F680 due to physical damages to the photosynthetic apparatus than those of intact cells.

Fluorescence changes due to physiological perturbations in a complex matrix such as a plant leaf may depend on several diverse biochemical and biophysical mechanisms. In general, the fluorescence magnitude changes in the blue-green regions of the spectrum on intact leaves followed by exposures to stress conditions have been mainly associated with changes in concentrations of fluorophores and/or photosynthetic pigment contents. These may also depend on a number of additional factors such as viscosity of the cytosolic solution, hydration state of membrane, membrane integrity, pH, temperature, oxidation and reduction states of the fluorophores, and membrane topology (Chappelle et al., 1991; Kim et al., 1997; Morales et al., 1998). Improved elucidation of mechanisms and factors governing the blue-green fluorescence changes should enhance the utilities of fluorescence techniques for the remote detection of the physiological state of plants.

3. Fluorescence Studies

Higher plants, in general, have similar multispectral fluorescence emission maxima (e.g., F440,F525, F685, F730) under UV excitation; however, the magnitudes of fluorescence at a given wavelength among many plant types and species are significantly different (1985). For instance, studies by Chappelle et al. (1985, 1987) showed that monocots can be differentiated by having a much higher F440 and F440/F685 ratio than that found in dicots. It was suggested that the differences in the fluorescence intensities between monocots (i.e., soybean) and dicots (i.e., wheat) especially in the blue-green region may be attributed to leaf anatomy and leaf-vein arrangements (Stober and Lictenthlaer, 1992). Further, the F440 difference between dicot and monocot species was thought to be due concentration difference in phenolics (Stober and Lictenthaler 1993a). Deciduous and coniferous trees also showed inherent differences throughout the spectrum (Chappelle et al., 1987).

While a number of different plant types exhibited unique fluorescence characteristics, fluorescence responses within a plant species are sensitive to changes in environmental conditions and are usually pre-visual (e.g., Stober and Lictenthaler, 1993b; Lictenthaler et al., 1991, 1996; Kim et al., 1998). Changes in fluorescence intensities as results of environmental perturbations are usually accompanied by alterations of physiological status and are wavelength and species dependent . Hence, multiple fluorescence ratios have been used as indicators of plant vigor. For instance, a combination of either blue or green to red or far-red, such as F440/F685, F440/F740, F525/F685, and F525/F740, have shown to be sensitive indicators of changes in physiological states of plants as results of environment perturbations (e.g., Chappelle et al., 1987; Lictenthaler, 1991, 1993, 1996; Lang et al., 1996). In addition, when laser is used as the excitation source, fluorescence ratios assist in normalizing out pulse to pulse variations in laser power (Chappelle et al., 1984a).

Photosynthesis is affected when the physiological and biochemical processes of plants are disturbed. Thus, when plants are subjected to those stress conditions that directly exacerbate the photosynthetic processes, these are manifested as increases in the Chl fluorescence emissions at F685 and F730 (e.g., Lictenthaler and Rinderle, 1988). However, some conditions affecting only certain biochemical/biophysical reactions and do not initially impact the integrity of photosynthesis, may be observed as changes in the blue-green region of the spectrum. Usually, increased blue-green fluorescence emissions have been observed as results of negative effects on physiological status of plants. Ultimately, the accumulative effects of this second type of condition will result in decreased photosynthetic efficiency or breakdown (or less synthesis) of photosynthetic pigments. Several investigations suggested that the use of a combination of either blue or green to red or far-red fluorescence ratio may be better indicators for the detection of pre-visual early stress symptoms than Chl fluorescence alone (Chappelle et al., 1987; Banninger et al., 1990; Lang et al., 1996; Lictenthaler et al., 1991, 1996; Stober and Lictenthaler, 1993b).

Water stress negatively affect many physiological processes in plants including photosyntheis, and thus Chl fluorescence can be highly responsive (Rosema et al., 1992). Furthermore, it has been shown that blue-green bands were highly responsive to water stress. For instance, potted soybean plant leaves withheld water for four days in the greenhouse showed higher fluorescence spectra throughout the spectrum (Chappelle et al., 1984b). In addition, F440/F530 ratio was significantly lower for water stress soybean plants. Chappelle and Williams (1987) also reported significantly increased F440, F525, F685, and F740 for potted cotton plants subjected to short term (2 days) mild

water stress with respect to well-watered plants. An increase in F440 on drying maple leaves, which the symptoms were not visually apparent, was observed (Theisen, 1988). Goulas et al. (1990) observed decreases in F440/F530 and F440/F685 ratios as spinach leaves become water stressed. They stated that the use of blue-green fluorescence improved the capabilities of stress detection in relation to plant water stress.

Significant increases in fluorescence intensities at F440, F525, F685, and F730 for tobacco plant leaves grown under a chronic-mild water stress were shown with the use of LIF imaging system (Lang et al., 1996). They noticed that the rate increases at F685 and F730 were much lower than those observed at F440 and F530. Fluorescence ratios F440/F685 and F440/F730 remained constant until leaf water content fell below 84 and 88% for green and aurea leaves, respectively; however, the ratios linearly increased as the water content decreased further. They observed rapid increases at F685 and F730 when the water stress was accompanied by a short-term (6 hours at 40°C) heat stress.

A relationship was found between fluorescence emissions at F440, F525, and F440/F730 ratio and the extent of red spruce (*Picea rubens* Sarg.) damage by acid deposition as the result of an increase in air pollutants generated by the high industrial density of the Northeastern United States (Chappelle and Williams, 1987). They reported that the magnitude of F440 related positively to the acid rain stress-index rankings, followed by F525, and F440/F730. In general, fluorescence emission peak at F685 were not clearly defined on coniferous species, however, samples from high acid-damage sites frequently showed high intensities at F685 band. Banninger et al. (1990) reported significant fluorescence differences for Norway spruce needles obtained from control and sites damaged by heavy metals in May, July, September, and November. They showed significant changes in the blue-green fluorescence emissions as functions of growth months and heavy metal damage. No significant differences were observed in the Chl fluorescence bands and the F685/F730 ratio between samples from heavy metal damage sites and undamaged sites; however they suggested that F440/F730 was a good indicator of heavy metal effects. Significant fluorescence differences were observed between heavy metal (i.e, Hg, Cd) treated and control sea grass leaves in the blue-green region of the spectrum (Cecchi et al., 1998). Fluorescence responses of sea grass showed a significant variations within the leaf as a function location from the base. Consequently, high doses of heavy metal treatments resulted lower F440 and F530 responses.

Differences in fluorescence emission intensities due to growth-light conditions can be seen in all four fluorescence emission bands. A study conducted by Kim et al., (1998) observed that fluorescence intensities at F440, F525, F685, and F730 for alder leaves grown in shaded regions were significantly higher than those grown in sunlit regions. The rates of P_n for the sunlit leaves were significantly higher than leaves grown under shaded regions. They speculated that the significant fluorescence differences between sunlit and shaded leaves may be due to differential concentrations of UV screening flavonol compounds. Stober and Lictenthaler (1993b) studied fluorescence responses of soybean and wheat plants grown for two weeks under different irradiance regimes ranging from 10 to 675 $\mu mol\ m^{-2}\ S^{-1}$ and 10 to 870 $\mu mol\ m^{-2}\ S^{-1}$, respectively. They observed nominal F440 decreases and significant F685 and F730 decreases as a function of increasing irradiance levels for both species. The results showed that F440/F685 ratio exhibited a strong correlation with the irradiance applied to both plant species; however, no significant changes in F685/F730 as a function of Chl concentrations from this experiment. Controlled environment growth chamber studies were conducted to determine the effects of UV-B irradiation on cucumber plants

(Sandhu et al., 1997). Elevated UV-B irradiation induced typical symptoms of plant physiological stress. In addition to physiological and morphological indicators of UV damage, UV-B exposed and non-UV exposed plants were clearly separated with fluorescence measurements. The blue-green fluorescence emissions showed increasing trends and the F525/F685 ratio increased significantly as the result of enhanced UV-B exposure. Subhash et al. (1995) reported that fluorescence ratios, F440/F525, F440/F730, and F525/F730 from *Salvia splendens* L. grown in elevated UV-B showed up to 40% decreases with respect to control plants.

Several studies have been conducted to demonstrate that sensitive fluorescence responses in relationship to nutrient status on corn. For instance, Chappelle et al. (1984a) showed that lack of K increased the fluorescence at 685 and 740 nm more than three fold along with a small decrease at 440 nm. They suggested that F685 and F740 responses were indicative of the role of K in maintaining g_s. A study conducted with LIF imaging system by Heisel et al. (1996) indicated that mineral deficiencies can be monitored by fluorescence ratios, and in some cases directly on single band images based on the spatial distribution of fluorescence emission. Nitrogen is the plant nutrient most essential for plant growth. As an essential constituent for amino acids, protein, nucleotide, and the tetrapyrolle ring of chlorophyll, deficiencies decrease the photosynthetic pigments concentrations and severe cases can be seen visually as yellowing of the leaves. Decreasing trends in fluorescence at F440, F525, F685, and F730 were observed on field grown corn leaves as the N fertilization levels increased from 0% to 150% of optimal fertilization level (McMurtrey et al., 1994). They observed increases in F440/F685, F440/F740, F525/F685, and F740/F685 ratios as a function of increasing N fertilization levels. Statistical analysis showed that F440/F685 had the most significant mean separation in terms of N fertilization levels. A subsequent study by Heisel et al. (1996) indicated that the F440/F685 and F440/F730 ratios were found to be more sensitive to N fertilization levels on field grown corn plants than F685/F740. A recent study conducted by Kim et al. (1997) demonstrated the sensitivity of fluorescence measurements to the effects of chronic exposure to combinations of moderately elevated levels of tropospheric CO_2 and O_3 on crop species. With the use of fluorescence imaging system capable of capturing images at F450, F550, F680, and F740 bands, they demonstrated that although visible stress symptoms such as chlorosis, discoloration, or necrosis were not evident in plant leaves, the fluorescence responses were statistically significant to the effects of moderately elevated O_3, elevated CO_2, and concomitant elevated O_3 and CO_2 environments. The most significant difference among the treatments were observed in the leaves grown under elevated O_3 in the absence of elevated CO_2 at F450 and F550. Fluorescence responses also demonstrated the partial negation of elevated O_3 effects by the CO_2 enriched environment.

4. Instrumentation

Several different types of instrumentation have been used for the steady-state fluorescence measurements involving intact plant materials. Principle components required for a fluorescence detection system include an excitation source consisting of a relatively high intensity monochromatic light and a photon detecting device. In general, two types of systems have been used in terms of the excitation light sources based on either a continuous wavelength (CW) light source or a high intensity pulsed light such as lasers to induce fluorescence from intact plant materials. Fluorescence emission

is then passed through either a monochrometer or a series of interference filters to remove reflected and scattered excitation radiation before reaching a photon detecting device.

A number of laboratory based fluorescence studies have been conducted with the use of commercially available spectro-fluorometers (e.g., Lang et al., 1996; McMurtrey et al., 1996) that utilize a CW light, e.g., xenon arc, quartz-halogen. These CW light sources provide stable fluorescence excitation. In general, a spectro-fluorometer is equipped with two double monochrometers which allow to obtain fluorescence excitation and emission spectra. A sensitive photon detecting device such as a photomultiplier tube (PMT) is used to detect high spectral resolution fluorescence emission. spectrum. However, these types of instrumentation have a very small instantaneous field of view (IFOV) with target leaf areas ranging from 0.35 cm^2 to .8 cm^2 (e.g., Lang et al., 1992; McMurtrey et al., 1996).

The lack of adequate excitation light sources with sufficient power previously limited the remote fluorescence studies on intact plant material. However, the advent of the lasers has provided an ideal excitation source for *in situ* fluorescence studies (e.g., Chappelle et al., 1984a, 1984b). Lasers emit coherent light with a very high degree of monochromaticity and intensity. This is necessary to induce measurable fluorescence from intact vegetation in the remote mode. Pulsed lasers, such as nitrogen and Nd:YAG, have the advantage of having high light energy emission with a short pulse duration time (e.g., <= 10 ns). This results in a time-dependent fluorescence emission. A high speed gated detector system (e.g., gate width of 5 to 100 ns) synchronized to laser pulses is capable of resolving fluorescence emission in the presence of ambient solar radiation before it decays. Fluorescence yield from intact leaves within 100 ns time scale with 10 mJ pulse excitation is approximately four to five orders of magnitude higher than scattered background solar radiation. Thus, a fast gated detector system which improves signal to noise ratio has been essential in the use of LIF techniques in the remote mode.

The LIF systems with the *in situ* remote sensing capabilities have been fabricated in laboratories with commercially available components. The very earlier airborne systems which were designed to measure only Chl fluorescence (i.e., detection of marine organisms) will not be included in this discussion (e.g., Kim, 1973; Hoge et al., 1983). A systems described by Chappelle et al. (1984a) utilized a nitrogen laser emitting at 337 nm operating at 30 Hz with an output power of 9 mJ per pulse with 10 ns pulse width. Fluorescence emission was collected by an f2.8, 180 mm telescope and focused on the entrance slit of a scanning monochrometer where fluorescence emission from 400 to 800 nm were scanned and detected with a gated PMT. Stober and Lichtenthaler (1992) used a nitrogen laser emitting at 337 nm with a pulse energy of 2.5 mJ which operated at 10 Hz with 10 ns pulse width. In addition, a gated intensified linear-array detectors (i.e., 150 channels) coupled with polychromators (spectrographs) were used to capture a fluorescence emission spectrum from 400 to 800 nm. These types of detection systems that are capable of capturing a full fluorescence spectrum induced by a single laser pulse in the presence of ambient radiation are referred to as optical multichannel analyser (OMA). Instead of linear-array diode detectors, the use of charge couple device (CCD) detector lines to acquire spectra have been reported (e.g., Szabo et al., 1992; Saito et al., 1998).

In conjunction with the use of OMA systems, other types of lasers with various pulse energies and repetition rates have been used. For instance, a van mounted fluorescence system by Cecchi et al. (1996, 1998) used a pulsed xenon-chloride excimer laser (308 nm) for blue-green emission in

which delivered approximately 80 mJ per pulse with 15 ns width at 10 Hz. In addition, a second excitation wavelength at 480 nm for Chl fluorescence was achieved by the use of a dye laser which was optically pumped by the excimer laser. Other investigations have used frequency-tripled Nd:YAG lasers which emit at 355 nm as the fluorescence excitation source (Johansson et al., 1996; Alder et al., 1995; Saito et al., 1998). Johansson et al. (1996) shifted their Nd:YAG laser output in a high pressure deuterium cell to generate radiation at 397 nm (Raman-shift) with 30 mJ pulse energy at 20 Hz. This excitation wavelength provided a higher Chl fluorescence yield and was less dangerous to eyes. Their system can acquired multispectral coarser resolution images of a large target (i.e., whole tree canopy) by "push-broom" method. A system described by Saito et al. (1998) also used a Nd:YAG with 0.2 mJ pulse energy (10 ns pulse width) at 7 Hz. They also used bifurcated fiber optics capable of transmitting the laser beam as well as the fluorescence emission. Morales et al. (1994) used a system using a nitrogen laser (337 nm) operating at .33Hz. The power output of the laser was only 30 μJ per pulse with 3 ns duration which in comparisons to other systems was considerably lower.

The majority of the LIF studies to date have used point-source measurements usually acquiring fluorescence spectra on small leaf areas. It was recognized that fluorescence emission across leaves are highly variable due to localized anatomical and physiological variations. This variability can be a function of numerous environmental factors. Advances in imaging technology provide a means to capture fluorescence images on a larger portion of plant leaves where variability across the leaf can be better characterized. One of the earlier use of fluorescence imaging was reported by Omasa and colleagues in 1987. However, they only measured Chl fluorescence induction kinetics at 685 nm induced by a filtered CW light. A system developed by Daughtry et al. (1997) also utilized a single broad-blue band to determine the fraction of crop residue cover where prior studies by McMurtrey et al. (1993) showed that senescent leaves or crop residues emit blue-green fluorescence under UV excitation. As the excitation source, they used CW UV-A fluorescent lamps filtered with a Schott glass band pass filter which provided a stable and homogeneous illumination on the target area. Kim et al. (1997) reported a multispectral imaging system using CW UV-A fluorescent lamps as the excitation source where images at the blue, green, red, and far-red region of the spectrum were captured.

Most recently, a LIF imaging system with multispectral capabilities have been reported (e.g., Lichtenthaler et al., 1996; Heisel et al., 1996; Sowinska et al., 1996; Lang et al., 1996). This imaging system was equipped with a frequency tripled Nd:YAG laser (355 nm) with adjustable repetition rate from .8 to 1 kHz emitting 10 μJ pulse energy with 100 ps pulse width. The laser beam was expanded to illuminate a 20 cm diameter leaf target area. A gated intensifier coupled to a CCD array with 288 X 384 elements provided up to 16 bit images. A filter wheel containing four band pass interference filters at the blue, green, red, and far-red bands with 10 nm full width at half maximum (FWHM) was used to sequentially capture fluorescence images. To produce a single band image which took 8 seconds, a composite of over 200 to 400 laser pulse were required (Heisel et al., 1996). The current state of technology lasers may have pulse to pulse peak power variations. In addition, a heterogeneous spatial intensity distribution of a laser pulse may also affect the quality of fluorescence especially when the beam is expanded to illuminate larger areas for imaging applications. The use of multiple fluorescence band ratios may circumvent these problems (Chappelle et al., 1984a) and thus, LIF ratios have been frequently used.

5. Summary

A number of significant findings have been made over the past few years using the laser induced fluorescence (LIF) active remote sensing technique. Laser induced fluorescence has successfully used for the early pre-visual detection of physiological changes in vegetation due to natural and anthropogenic factors. Several studies have demonstrated that fluorescence emissions of plant leaves are sensitive both in magnitude and wavelength, to the changes in nutrient and soil moisture availabilities, and to the effects of elevated levels of environmental pollutants such as heavy metals, acid deposition, tropospheric ozone, and UV-B radiation. Major species types were also differentiated on the basis of their unique fluorescence spectra. Algorithms have been developed using ratios of certain fluorescence bands which allowed the remote estimation of the relative rate and efficiency of photosynthesis. Further, studies conducted with fluorescence imaging enabled spatial characterizations to be made over areas which cannot be obtained with line spectrum point sampling techniques. It has also been demonstrated that experimental errors and variances associated with line spectrum measurements can be significantly reduced with imaging techniques.

The success of previous fluorescence research leads us to recommend further rigorous investigations into the use of LIF as a viable canopy level remote sensing technique. Passive reflectance techniques at this time are limited in their ability to detect subtle changes in the concentration and oxidation states of the many compounds involved in the light reactions of photosynthesis. Knowledge of these changes we consider to be fundamental in the remote assessment of both the rate and efficiency of photosynthesis and also the early detection of stress damage. Fluorescence sensing has potential for detecting electron transfer efficiency during photosynthesis. The use of the canopy level LIF measurements in conjunction with current passive reflectance measurements will greatly enhance the overall value of remote sensing in the study of vegetative ecosystems.

References

Albers, B., J. DiBenedetto, S. Lutz, and C. Purdy (1995), More Efficient Environmental Monitoring with Laser-Induced Fluorescence Imaging, *Biophotonics International*, pp. 42-54

Agati, G., P., Mazzinghi, P. Fusi, F. and Ambrosini, I. (1995), The F685/F730 chlorophyll fluorescence ratio as a tool in plant physiology: response to physiological and environmental factors, *J. Plant Physiol.*, 145: 228-238

Banninger, C., Chappelle, E.W., McMurtrey, J.E. (1990), Variations in Fluorescence of Stressed and Non-stressed Norway Spruce Needles over the Growing Season, Int. Geoscience and Remote Sensing Symp., IGARSS'90 Digest, Vol 3, 1931-1937

Cecchi, G., Kim, M.S., Bazzani, M., et al (1998), Fluorescence responses of Mediterranean sea grass *Posidonia Oceanica*: Summer 1997 ATOM - LIFT Campaign, SPIE Vol 3382:126-132

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

Cerovic, Z., Morales, F., Moya, I. (1994), Time-resolved spectral studies of blue-green fluorescence of leaves, mesophyll and chloroplasts of sugar beet (*Beta Vulgaris* L.), *Biocimica et Biophysica Acta*, 58-68

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

Chappelle, E.W, McMurtrey, J.E, Wood F.M., and Newcomb, W.W. (1984b), Laser induced fluorescence of green plants 2. LIF changes caused by nutrient deficiencies in corn, *Appl. Optics*, vol 23 139-134

Chappelle, E.W., Wood, F.M., Newcomb, W.W., and McMurtrey III, J.E. (1985), Laser induced fluorescence of green plants. 3. LIF spectral signatures of five major plant types, *Appl. Optics*, Vol. 24, pp. 74-80, 1985.

Chappelle EW, Williams D.L. (1987), Laser induced fluorescence(LIF) from plant foliage, *IEEE Trans Geosci. Remote Sensing*, Vol GE-25 pp 726-736

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

D'Ambrosio, N., Szabo, K, and Lichtenthaler, H.K. (1992), Increase of the chlorophyll fluorescence ratio F690/F735 during the autumnal chlorophyll breakdown, *Radiant Environ. Biophys.*, 31:51-62

Daughtry, C.S.T., J.E. McMurtrey, M.S. Kim, and E.W. Chappelle (1997), Estimating Crop residue Cover by Blue Fluorescence Imaging, *Remote Sens. of Environ.*, 60:14-21.

Goulas, Y.I, Moya, I., and Schmuck, G. (1990), Time resolved spectroscopy of the blue fluorescence of spinach leaves, *Photosynth. Res.* 25. 299-307

Hagg, C., Stober, F., Lichtenthaler, H.K. (1992), Pigment content, chlorophyll fluorescence and photosynthetic activity of spruce clones under normal and limited nutrition, *Photosynthetica*, 27 (3):385-400

Harris, P.J, and Hartley, R.D. (1976), Detection of bound ferulic acid in cell walls of the graminae by ultraviolet fluorescence microscopy, *Nature* 259, 508-510

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

Hoge, F.E., Swift, R.N., and Yungel, J.K., (1983) Feasibility of airborne detection of laser induced fluorescence from green plants, *Appl. Optics*, Vol. 22: 2991-2998

Horton P, Ruban, A.V., Walters, R.G., (1994) Regulation of light harvesting in green plant: Indication by nonphotochemical quenching of chlorophyll fluorescence, *Plant Physiol.*, 106:415-420

Johansson, J., Anderson, M., Edner, H., Mattson, J., and Svanbuerg, S., (1996), Remote fluorescence measurements of vegetation spectrally resolved and by multi-colour fluorescence imaging, *J. Plant Physiol.*, Vol. 148, 632-637

Kim, H.H. (1973) New Algae Mapping Technique by the Use of an Airborne Laser Fluoresensor, *Applied Optics*, Vol. 12, 1454-1459

Kim, M.S., Cecchi, G., Chappelle, E.W., (1998) Fluorescence responses and photosynthetic rates of sunlit and shaded leaves of Italian alpine forest species: Summer 1997 ATOM - LIFT campaign. SPIE Vol 3382:118-125

Kim, M.S., C.L. Mulchi, C.S.T. Daughtry, E.W. Chappelle, J.E. McMurtrey, (1997) Fluorescence Images of Soybean Leaves Grown Under Increased Ozone, Advances in Laser Remote Sensing for Terrestrial and Oceanographic Applications, SPIE Vol: 3059, pp. 22-31.

Kim, M.S., D. Krizek, E.W. Chappelle, C.S.T. Daughtry, J.E. McMurtrey, R. Sandhu, L.A. Corp, and E. Middleton, (1996), Fluorescence Imaging System (FIS): Assessment of Vegetation Stress, European Symposium on Satellite Remote Sensing III, Taormina, Italy, SPIE Vol: 2959: 4-13

Kok, B. (1965), Photosynthesis, the pathway of energy: In *Plant Biochemistry*, J. Bonner and J.E. Varner (eds.), pp. 903-930, Academic Press

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

Lang, M., Stiffel, P., Braunova, Z., and Lichtenthaler, H.K. (1992), Investigation of the Blue-green Fluorescence emission of plant leaves, *Bot. Acta*, 105, 395-468

Lang, M., Lichtenthaler, H.K., Sowinska, M., Heisel, F., and Miehe, J.A. (1996), Fluorescence imaging of water and temperature stress in plant leaves, *J. Plant Physiol.*, 148:613-621

Lavorel J, Etienne AL (1977) *In vivo* chlorophyll fluorescence. *In Primary Processes in Photosynthesis*, eds. J. Barber, pp 203-268, Elsviser Amsterdam

Lictenthaler H.K. and Rinderle U (1988) Role of chlorophyll fluorescence in the detection of stress conditions of plants. CRC Critical Rev Anal Chem 19:29-85

Lichtenthaler, H.K., Lang, M., Stober, F., (1991) Nature and variation of the blue fluorescence spectra of terrestrial plants, Proc. Internat. Geoscience and Remote Sens. Symp. pp. 2283-2286

Lichtenthaler, H.K., (1993), Studies on the constancy of the blue and green fluorescence yield during the chlorophyll fluorescence induction kinetics (Kaustsky effect), *Radiant Environ. Biophys.*, 32:357-365

Lichtenthaler, H.K., Lang, M., M. Sowinska, F. Heisel, and J.A. Miehe (1996), Detection of Vegetation Stress Via a New High Resolution Fluorescence Imaging System, *J. Plant Physiol.*, 148:599-612

McMurtrey, J.E., Chappelle, E.W., Daughtry, C.S.T., and Kim, M.S. (1993), Fluorescence and Reflectance of Crop Residue and Soil, *J. Soil and Water Conservation*, 48 (3):207-213, 1993

McMurtrey J.E, Chappelle E.W, Kim M.S., et al. (1994) Distinguishing Nitrogen Fertilization Levels in Field Corn (*Zea mays* L.) with Actively Induced Fluorescence and Passive Reflectance Measurements, *Remote Sens Environ.*, 47:36-44

Mohammed, G.H., Binder, W.D., and Gillies, S.L. (1995), Chlorophyll Fluorescence: A Review of its practical Forestry Applications and Instrumentation, *Scan. J. For. Res.*, 10:383-410

Morales, F., Cerovic, Z.G., Moya, I. (1998), Time-resolved blue-green fluorescence of sugar beet leaves. Temperature-induced changes and consequences for the potential use of blue-green fluorescence as a signature for remote sensing of plants, *Aust. J. Plant Physiol.*, 25:325-334

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

Omasa, K., Shimazaki, K.I., Aiga, I., et al (1987), Image analysis of chlorophyll fluorescence transients for diagnosing the photosynthetic system of attached leaves, *Plant Physiol.*, 84:748-7529

Rosema, A., Cecchi, G., Pantani, L., Radicati, M., Romoli, M., Mazzinghi, P., Van Kooten, O., and Kliffen, C., (1992) Monitoring photosynthetic activity and ozone stress by laser induced fluorescence in trees, *Int. J. Remote Sensing*, vol 13, no.4, 737-751

Saito, Y., Takahashi, K., Nomura, K., Mineuchi, K, Kawahara, T.D., Nomura, A., Kobayashi, S., and Ishii, H., (1997), Visualization of a laser-induced fluorescence of plants influenced by environmental stress with a microfluorescence imaging system and a fluorescence lidar system., SPIE, Vol 3059, pp. 1990-198

Schweiger, J., Lang, M., and Lichtenthaler, H. (1996), Differences in fluorescence excitation spectra of leaves between stressed and non-stressed plants, *J. of Plant Physiol.*, Vol. 148 536-547

Sowinska, M., Heisel, F., Miehe, J.A., Lang, M., Lichtenthaler, H.K., and Tomasini, F. (1996) Remote Sensing of Plants by Streak Camera Lifetime Measurements of the Chlorophyll *a* Emission, *J. Plant Physiol,* 148:638-644

Stober, F., and Lichtenthaler, H.K., (1992), Changes of the Laser-Induced Blue, Green and Red Fluorescence Signatures during Greening of Etiolated Leaves of Wheat, *J. Plant Physiol.,* Vol. 140:673-680

Stober, F. and Lichtenthaler, H.K., (1993a), Characterization of the laser induced blue, green, and red fluorescence signatures of leaves of wheat and soybean grown under different irradiance, *Physiologia Plantarum*, 88:696-704

Stober, F. and Lichtenthaler, H.K. (1993b), Studies on the Localization and Spectral Characteristics of the Fluorescence Emission of Differently Pigmented Wheat Leaves, *Bot. Acta*, 106:365-370

Stober, F. Lang, M., and Lichtenthaler, H.K. (1994), Studies on the blue, green, red fluorescence signature of green etiolated and white leaves, Remote Sens. Environ., 47:65-71

Subashi, N., Mazzinghi, P., Agati, G., Fusi, F., and Lercari, B. (1995), Analysis of Laser-induced line shape of intact leaves: Application to UV stress detection, *Photochem. and Photobiol.*, Vol. 62 (4): 711-718

Szabo, K., Litenthaler, H.K., Kocsanyi, L., and Ritchter, P. (1992), A CCD-OMA device for the measurement of complete chlorophyll fluorescence emission spectra of leaves during the fluorescence induction kinetics, *Radiant Environ. Biophys.*, 31:153-160