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Fluorescence sensing systems: In vivo detection of biophysical variations in field corn due to nitrogen supply

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Abstract

Leaf and canopy fluorescence properties of field corn (*Zea mays* L.) grown under varying levels of nitrogen (N) fertilization were characterized to provide an improved N sensing capability which may assist growers in site-specific N management decisions. In vivo fluorescence emissions can occur in the wavelength region from 300 to 800 nm and are dependent on the wavelength of illumination. These light emissions have been grouped into five primary bands with maxima most frequently received from corn at 320 nm (UV), 450 nm (blue), 530 nm (green), 685 nm (red), and 740 nm (far-red). Two active fluorescence sensing systems have been custom developed; a leaf level Fluorescence Imaging System (FIS), and a canopy level Laser Induced Fluorescence Imaging System (LIFIS). FIS sequentially acquires high-resolution images of fluorescence emission bands under darkened laboratory conditions, while LIFIS simultaneously acquires four band images of plant canopies ≥ 1 m^2 under ambient sunlit conditions. Fluorescence emissions induced by these systems along with additional biophysical measures of crop condition; namely, chlorophyll content, N/C ratio, leaf area index (LAI), and grain yield, exhibited similar curvilinear responses to levels of supplied N. A number of significant linear correlations were found among band emissions and several band ratios versus measures of crop condition. Significant differences were obtained for several fluorescence band ratios with respect to the level of supplied N. Leaf adaxial versus abaxial surface emissions exhibited opposing trends with respect to the level of supplied N. Evidence supports that this confounding effect could be removed in part by the green/blue and green/red ratio images. The FIS and LIFIS active fluorescence sensor systems yielded results which support the underlying hypothesis that leaf and canopy fluorescence emissions are associated with other biophysical attributes of crop growth and this information could potentially assist in the site-specific management of variable-rate N fertilization programs.

Keywords: Fluorescence imaging; Carbon; Nitrogen; Precision agriculture; *Zea mays* L.

1. Introduction

In foliage, a large fraction of the in vivo nitrogen (N) pool is allocated to processes and cell structures related to carbon (C) assimilation. Adequate N availability for growth and seed production in crops is ensured by application of N-enriched fertilizers. Corn (*Zea mays* L.), a C_4 species, has a higher requirement for N than most other crops. Although N is needed by the corn plant throughout the growing season, N uptake from the soil is greatest during the period of most rapid growth; this extends from 2 to 3 weeks after plant emergence until tasseling. Small grains, typically C_3 species, are likewise responsive to N application but their total requirements are considerably less than corn. However, excessive N application may be accompanied by reductions in yield and is a primary source of NO_3^- pollution delivered to ground and surface waters, and as a consequence, fertilization should be adjusted to provide adequate but not excessive amounts of N (Wood, Reeves, & Himelrick, 1993). Currently, the N application rates chosen by producers are influenced by several factors, including: soil and

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tissue N assays, N fertilizer performance, and expected yields. Since the actual uptake of N by crops is greatly influenced by perturbations in environmental conditions, in particular the interaction of moisture and temperature, there is considerable room to improve current methods to provide practical, reliable and quantitative techniques for evaluating crop N utilization to optimize N application (Doerge, 2002).

Actively induced fluorescence technologies offer spectral sensing methods with potential for the remote assessment of N uptake in crops (Corp, McMurtrey, Chappelle, Daughtry, & Kim, 1997; Heisel et al., 1997; Heisel, Sowinska, Miehe, Lang, & Lichtenthaler, 1996; Langsdorf et al., 2000; McMurtrey, Chappelle, Kim, Mesinger, & Corp, 1994). The principle of fluorescence involves the absorption of a specific wavelength of light by a fluorophore followed by the dissipation of the absorbed energy by light emission at longer wavelengths within a very short (<200 ns) period of time. In vivo fluorescence emissions from vegetation occur throughout the ultraviolet to visible regions of the spectrum, with maxima most frequently occurring in the UV-A, blue, green, red, and far-red (Chappelle, Wood, McMurtrey, & Newcomb, 1984; Corp et al., 1996, 1997; Johnson, Mantha, & Day, 2000). The UV-A fluorescence emission band can only be observed when plants are irradiated with UV-B (~ 280 nm) and has been attributed to rubisco and additional plant proteins which contain aromatic amino acids (Corp et al., 1997). The overlapping blue and green bands are a convolution of fluorescence emissions originating from several plant constituents (Chappelle, McMurtrey, Wood, & Newcomb, 1984; Chappelle, Wood et al., 1984; Lang, Stober, & Lichtenthaler, 1991). The majority of the static blue fluorescence, that is variations which occur over days to weeks, has been attributed to hydroxycinnamic acids, primarily ferulic acid covalently linked to the leaf epidermis and cell walls (Lichtenthaler & Schweiger, 1998). Additional blue and green fluorescent compounds can have smaller but more dynamic contributions (i.e., diurnal variations) to the in vivo fluorescence emission spectrum (Cerovic, Samson, Morales, Tremblay, & Moya, 1999; Chappelle et al., 1999). Chlorophyll fluorescence emissions occur in the red and far-red regions of the spectrum and have been extensively explored and relationships have been established to pigment contents and plant primary metabolism (Gitelson, Buschmann, & Lichtenthaler, 1998; Mohammed, Binder, & Gillies, 1995; Rosema, Snel, Zahn, Buurmeijer, & Van Hove, 1998; Valentini et al., 1994).

Several investigators have demonstrated relationships between fluorescence emission bands or band ratios to plant health and growth condition (see reviews, Buschmann, Langsdorf, & Lictenthaler, 2000; Cerovic et al., 1999; Chappelle et al., 1999). Fluorescence measurements have shown a great deal of promise in the remote assessment of the relative impact of environmental factors, including: nutrient supply in crop canopies (Corp et al., 1997; Heisel et al., 1997, 1996; Langsdorf et al., 2000; McMurtrey et al., 1994, 2002), differentiation of crops and trees grown under controlled exposures of elevated O_3 (Kim et al., 2001; Rosema et al., 1998), irradiance level (Richards, Schuerger, Capelle, & Guikema, 2003), UV-B exposure (Krizek, Middleton, Sandhu, & Kim, 2001; Middleton, Chappelle, Cannon, Adamse, & Britz, 1996), and quantifying the amount of crop residue covering agricultural soil surfaces (Daughtry et al., 1995; McMurtrey, Chappelle, Kim, Mesinger, & Corp, 1993). Currently, several research groups are using fluorescence sensing systems operating from a variety of platforms to receive fluorescence information and are relating this information to biological activity in both terrestrial and aquatic ecosystems (Buschmann & Lictenthaler, 1998; Cecchi et al., 1994; Lichtenthaler, Lang, Sowinska, Heisel, & Miehe, 1996; Ounis, Cerovic, Briantais, & Moya, 2001).

The objectives of the current study were to: (1) determine the regions of the fluorescence emission spectrum from corn leaves that are sensitive to changes in N supply; (2) establish relationships among fluorescence emission bands and band ratios to biophysical measures of leaf and crop productivity; and (3) evaluate whether these relationships can be detected at canopy levels of fluorescence sensing. The results from this study provide considerations for future design and development of fluorescence sensing instrumentation and measurement protocols. These fluorescence investigations are ultimately intended to provide information that can be incorporated into prescription algorithms for site-specific variable applications of N containing fertilizers for crop production.

2. Methods and materials

2.1. Experimental design and plant growth conditions

Field corn sites were prepared by conventional tillage methods which incorporated the annual recommended soil test rate to provide optimal available phosphorus (P) and potassium (K). The pH of the field maintained at 6.9 with dolomitic lime which also supplied the essential minerals, calcium (Ca) and magnesium (Mg), while other essential nutrients were supplied by the natural mineralization of the parent soil. N treatments, supplied through a variable rate application of urea, were selected to provide plant growth conditions ranging from classical symptoms of N deficiency to physiological conditions consistent with excess N supply as determined by the University of Maryland Soil Testing Laboratory. Measurements span two field sites located at the USDA Agricultural Research Center in Beltsville, MD.

Site #1—Corn (*Z. mays* L., 'Southern States 812') was grown in 0.77-m-wide rows at a stand density of 20,000 plants per ha on a sandy loam soil from the 1997 through 2000 growing seasons. The experimental design was a randomized complete block with four blocks, each containing eight N treatments (32 experimental units). Each treatment plot was 9.2-m-wide (containing 12 rows) by 4.6 m long and the treatments were 244, 203, 162, 122, 81, 41, 20,

and 0 kg N/ha. Measurements reported from this site were obtained during the 1998 and 2000 growing seasons.

Site #2—This site was part of a multi-disciplinary project entitled Optimizing Production Inputs for Economic and Environmental Enhancement (OPE). Corn (*Z. mays* L., 'Pioneer 33A14') was grown in 77-cm-wide rows at a stand density of 10,000 plants per hectare on a sandy loam soil for the 2001 growing season. The experimental design was a randomized complete block with three blocks, each containing four N treatments (12 experimental units). Each treatment plot was 18.2 m wide (containing 24 rows) by 28.3 m long and the treatments were 210, 140, 70, and 28 kg N/ha. Measurements reported from this site were obtained during the 2001 growing season.

2.2. Leaf and canopy measurements

Biophysical measurements spanned leaf to canopy levels, representing two field locations, three growing seasons, and several growth stages. Fluorescence measurements at Site #1 in 1998 were primarily focused at the leaf level on adaxial surfaces, with canopy fluorescence measurements captured over this site in 2000. Measurements were extended to a second location (Site #2) in 2001 where additional fluorescence characterizations were performed including emission comparisons of adaxial (upper) and abaxial (lower) leaf surfaces.

Measurements at Site #1 in 1998 were carried out in three collection intervals when the plants were at the following growth stages; V12 (12th leaf fully expanded, 45 days from emergence), VT (tasseling, 68 days from emergence), and R3 (grain development at the milk stage, 94 days from emergence). At the V12 stage, the 12th leaf was selected for all leaf level observations while the 4th leaf down from the flag leaf was selected for measurement at the VT and R3 stages.

2.2.1. Fluorescence spectra

The relative magnitudes and number of emission bands received from vegetation will vary with excitation wavelength. The fluorescence excitation and emission matrix (EEM) is a three-dimensional data matrix with the horizontal coordinates corresponding to excitation and emission wavelengths while the vertical coordinate corresponds to the intensity of light emission. In vivo corn leaf fluorescence emissions were determined in the wavelength range from 300 to 800 nm, while excitation characteristics were determined in the wavelength range from 250 to 700 nm.

A spectrofluorometer (Fluorolog II,[1] Spex Industries, Edison NJ) was used to collect the EEMs. The Fluorolog II utilizes two 0.22-m double spectrometers with gratings of 1200 grooves/mm. A 450-W xenon lamp was attached to the excitation spectrometer with entrance and exit slits set to 3 mm, yielding a 5.1-nm bandpass for excitation radiation. Changes in lamp intensity as a function of wavelength were normalized by using a beam splitter to deliver a portion of the excitation radiation to a calibrated silicon photo-diode. The emission spectrometer was attached to a photon-counting photomultiplier tube corrected to obtain linearity throughout the emission wavelength range of 290–850 nm. The entrance and exit slits for the emission spectrometer were set to 1 mm allowing a 1.7-nm bandpass. Photomultiplier voltage readings were calibrated to photon counts per second (cps). Leaf samples were held in place by a nonfluorescent anodized aluminum solid sample holder.

2.2.2. Fluorescence imaging system

A dark room Fluorescence Imaging System (FIS) was custom-fabricated to quantify leaf fluorescence emissions in four discrete bands (Kim et al., 2001). The UV excitation source consisted of four 15-W longwave UV-A fluorescent lamps (Model XX-15A, Spectroline, USA) arranged 0.2 m above the sample surface at 45° facing a central target area and provided nearly uniform broad-band illumination (3.3 W/m^2) centered at 365 nm. The radiation from the UV lamps was filtered with Schott UG-1 glass to eliminate radiation >400 nm. The detection system consisted of a thermoelectrically cooled (-15 °C) charge-coupled device (CCD) camera (Lynxx-2, SpectraSource Instruments, Westlake Village, CA, USA) capable of capturing 12-bit, 196×165 pixel images. The camera was coupled to an automated filter wheel (AB300, CVI Laser, USA) which contained four 10-nm full width at half maximum (FWHM) band pass interference filters centered at 450 nm (blue band), 550 nm (green band), 680 nm (red band), and 740 nm (far-red band). The camera's responsivity and variation due to nonuniform illumination for the system were calibrated using a flat field fluorescent target. For each image, dark current responses of the camera were subtracted and thermal corrections for the CCD camera were applied. For 2001, an intensified thermoelectrically cooled (-40 ± 0.1 °C) CCD camera capable of capturing 16-bit, 1036×1032 pixel image (Orbis-2, SpectraSource Instruments) was used.

2.2.3. Laser Induced Fluorescence Imaging System

The Laser Induced Fluorescence Imaging System (LIFIS) uses a high-intensity fixed emission solid-state laser and a fast-gated CCD detection system to differentiate fluorescence emissions from ambient solar radiation. The spatial variability in fluorescence emissions can be captured from vegetation in vivo without disturbing, moving, or excising plant material from its natural environment. A frequency-tripled Nd:YAG laser (INDI Quanta-Ray, Spectra-Physics, Mountain View, CA, USA) provided the excitation radiation. The laser was operated at 10 Hz emitting up to 125 mJ of 355 nm radiation per 7 ns pulse. The laser beam was manipulated at its exit point with an expanding

[1] Company and trade names are given for the benefit of the reader and do not imply any endorsement of the product or company by Science Systems and Applications, National Aeronautics and Space Administration, or the U.S. Department of Agriculture.

divergent lens system that enabled an adjustable area of illumination. The excitation energy density at the plant did not exceed 100 mJ/m^2 (Rosema & Zahn, 1997). Fluorescence emissions were captured by a 12-bit two stage peltier forced air cooled (12 °C) CCD camera which provided a super VGA resolution of 1024 (H) × 1280 (W) pixels (DiCam-PRO, Cooke, Auburn Hills, MI, USA). The camera was coupled through an ultra speed F2.5/105 mm distortion free tandem lens to a fast-gated 25-mm P20 phosphor screen image intensifier. Fluorescence emissions were collected within an 80-ns exposure window following each laser pulse. Operation within this time domain greatly reduced interference from ambient solar radiation. A multispectral lens was attached to the camera and separated four spectral images spatially on the two-dimensional detector array. The four spectral images were acquired simultaneously through four 10-nm FWHM band pass interference filters centered at 450, 550, 680, and 740 nm, comparable to the fore mentioned FIS system. A Cannon F1:1.6/11−110 mm electronic TV zoom lens was attached in front of the multispectral lens. LIFIS is entirely computer controlled via a 132-Mbyte burst rate PCI interface board connected to the camera through a 10-m fiber optic link.

2.2.4. Supporting measurements

Leaf chlorophyll *a*, chlorophyll *b*, and total carotenoid concentrations were determined. Freshly cut leaf disks (2.54 cm^2) were placed in 3.5 ml of dimethyl sulfoxide (DMSO) and sealed for 36 h at 25 °C. Absorption spectra were obtained using a dual beam spectrophotometer (Lambda 40, Perkin-Elmer, Norwalk CT, USA) and pigment concentrations were determined by equations developed for DMSO by procedures outlined by Lichtenthaler (1987). The leaf remainder, after disk removal, was freeze-dried at −45 °C, and ground to pass a 1-mm mesh. Total leaf carbon (C) and N determinations for leaf samples were obtained by the Dumas combustion method (Bellomonte, Constantini, & Giammarioli, 1987).

The leaf area index (LAI) of the vegetative canopy was measured at V12, VT, and R3, with the LAI 2000 Plant Canopy Analyzer (LI-cor, Lincoln, NE, USA). Six sets of LAI measurements (a single above canopy and four below canopy) were acquired at random locations throughout the treatment plots. Grain yields were based on hand harvested samples obtained from three 3 m^2 areas per plot at locations where fluorescence, chlorophyll concentrations, and foliar N determinations were obtained. Grain samples were oven dried at 50 °C prior to weighing.

2.3. Data analysis

Images acquired from FIS and LIFIS were processed using ENVI and IDL software (RSI, Boulder, CO, USA). Image intensity thresholds were determined to differentiate plant material from background, and descriptive statistics were calculated. Additional processing steps were required for LIFIS imagery, where spectral bands were extracted from each quadrant of the raw image. Ratio images were achieved by co-registration and warping based on an interactive selection of control points, and then applying band math expressions.

Orthogonal polynomial contrasts were utilized to identify the order (i.e., linear, quadratic, cubic) of the regression equations which best described the leaf, crop, and fluorescence biophysical responses as a function of N fertilization level. The coefficients for each regression equation were determined by an iterative nonlinear regression technique, and the maxima for each of the measured parameters were isolated by finding the zero solution of the first derivative from corresponding regression equations.

A mixed model analysis of variance (SAS, Cary, NC, USA) was used to assess the separability of plant parameters and fluorescence features with respect to N fertilization rate. The fixed effects for the mixed model was the N fertilization rate while the heterogeneity associated with the blocked field plots was designated as a random source of variation. LSD mean separations were deemed significant at $p \le 0.05$. Correlation analysis was used to identify linear relationships among measures of plant growth condition, fluorescence bands, and fluorescence band ratios.

3. Results

3.1. Plant growth analysis

For varying levels of N fertilization, leaf chlorophyll, leaf N/C ratio, canopy LAI, and crop grain yield were positively correlated indicating a common response among the measures of plant growth condition to the N fertilization regimes. Correlations between chlorophyll and N/C content were strong throughout the growing season ($r \ge 0.83$, $p < 0.01$). Chlorophyll was most strongly correlated with LAI during vegetative growth ($r = 0.87$, $p < 0.01$), but correlations to yield were stronger in the later growth stages VT ($r = 0.85$, $p < 0.01$) and R3 ($r = 0.88$, $p < 0.01$). The other three param-

Table 1
Corn grain yields for Site #1 in response to varying rates of N fertilization

Applied N (kg/ha)	1998 yield (kg/ha)	2000 yield (kg/ha)
244	9208 b*	10068 b
203	10300 ab	10229 b
162	10480 a	11485 a
122	10318 ab	9798 b
81	7753 c	8175 c
41	5799 d	6522 d
20	4662 d	5810 de
0	2495 e	4636 e

Means ($n = 12$) are based on four replicates with three sub-samples each.

*Means with the same letter are not significantly different by $LSD_{0.05}$ comparisons within year.

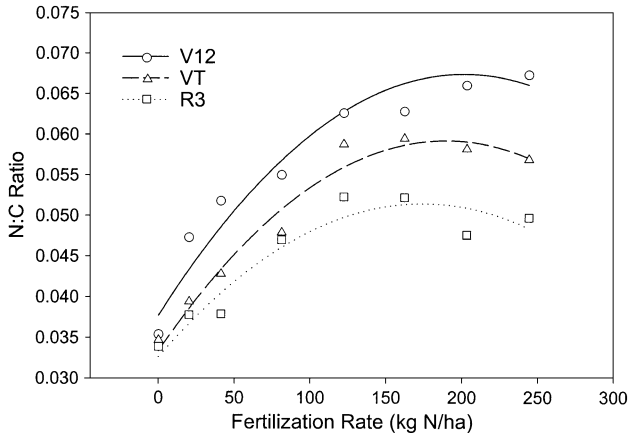

Fig. 1. Response of corn leaf N/C ratio to varying rates of N fertilization (1998). Each symbol represent a treatment mean ($n=12$) while each line was generated by a quadratic regression analysis ($n=96$). The N/C curvilinear response as a function of growth stage is described by the following equations: V12, $y=0.038+2.96e^{-4}x-7.33e^{-7}x^2$ ($r^2=0.90$, $S_e=0.0038$); VT, $y=0.033+2.73e^{-4}x-7.21e^{-7}x^2$ ($r^2=0.84$, $S_e=0.0042$); R3, $y=0.033+2.16e^{-4}x-6.21e^{-7}x^2$ ($r^2=0.53$, $S_e=0.0069$).

eters (Chl, N/C, LAI) were all well-correlated to yield at the VT stage ($r=0.85-0.87$, $p<0.01$).

The grain yield response to N is a key factor in precision farming systems. Significant reductions in yield were noted for fertilization rates above and below the prescribed optimal fertilization rate of 162 kg N/ha (Table 1). Grain yield as a function of N application rate can be described for the 1998 season by the equation: $y=2622+91.32x-0.2631x^2$ ($r^2=0.81$, $S_e=1250$), and for the 2000 season: $y=4449+65.88x-0.1747x^2$ ($r^2=0.85$, $S_e=990$). These equations predict that the maximum grain yield for Site #1 was achieved at a fertilization rate of 174 kg N/ha in 1998 and 189 kg N/ha in 2000. LAI measurements indicated that the crop had reached maximum light interception and biomass accumulation at VT, while slight decreases in LAI at R3 were indicative of N mobilization for reproductive development. LAI means were similar for N fertilization rates ≥ 122 kg N/ha, whereas significant decreases were obtained for treatments ≤ 81 kg N/ha as determined by the $LSD_{0.05}$ mean comparison procedure.

The response of foliar chemistry to N fertilization level was also quadratic, with chlorophyll, N, and N/C contents decreasing as the crop progressed through reproductive to grain fill growth stages. The calculated N rate associated with maximum N/C accumulation was as follows: V12, 0.068 N/C at 201 kg N/ha; VT, 0.059 at 189 kg N/ha; R3, 0.051 N/C at 174 kg N/ha (Fig. 1). Similar nonlinear results were obtained for chlorophyll as plants mobilized foliar N for reproductive functions during VT and R3 phases. Means for foliar chlorophyll, N, and N/C were similar for N fertilization rates ≥ 122 kg N/ha, whereas significant decreases were obtained for treatments ≤ 81 kg N/ha as determined by the $LSD_{0.05}$ mean comparison procedure. These findings indicate that the selected levels of N fertilization were effective in creating a range of plant growth conditions consistent with N deficiency to super-optimal N supply.

3.2. Fluorescence spectral characterization of field corn

The fluorescence EEMs acquired from optimally fertilized (Fig. 2) and N deficient corn adaxial leaf surfaces

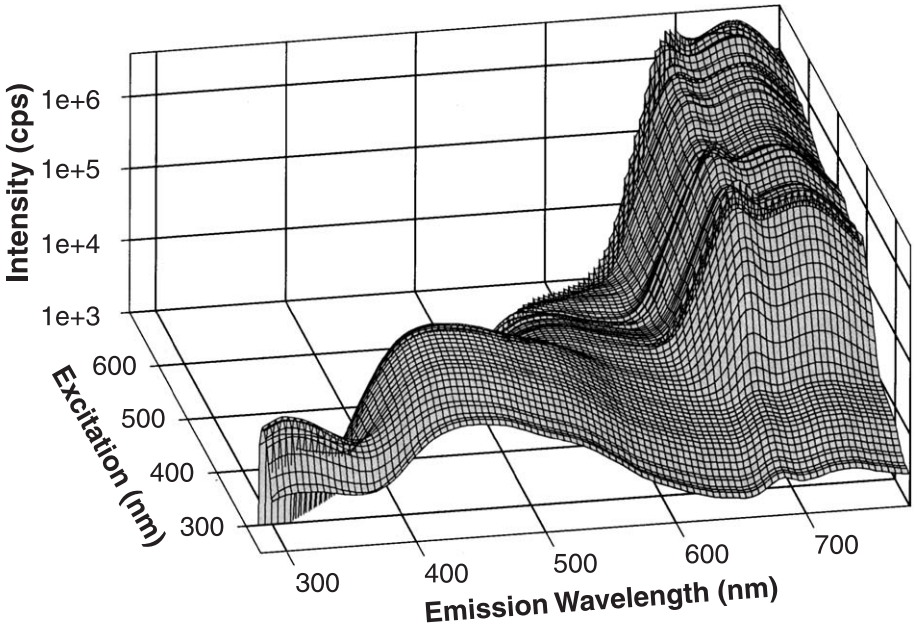

Fig. 2. Fluorescence Excitation and Emission Matrix (EEM) for a field corn adaxial leaf surface. Fourth from terminal leaf was excised at the VT stage from a 162 kg N/ha treatment plot (Site 1, 1998). In vivo fluorescence emissions occur in UV through visible regions of the spectrum.

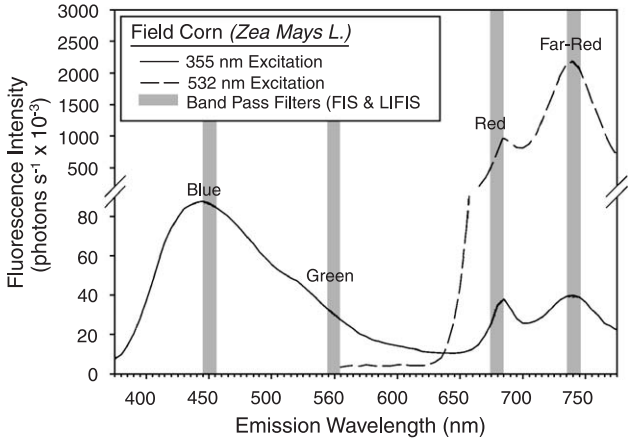

Fig. 3. Fluorescence emission spectra from the adaxial leaf surface of field grown corn acquired with a spectrofluorometer at two excitation wavelengths corresponding to the frequency tripled ($\lambda = 355$ nm) and frequency doubled ($\lambda = 532.5$) Nd:YAG laser emissions.

indicated the following highly conserved emission maxima; in the UV at 320 nm with $\lambda_{ex\text{-}max} = 280$ nm, the blue at 450 nm with $\lambda_{ex\text{-}max} = 350$ nm, and in the green (530 nm), red (684 nm), and far-red (738 nm) all with $\lambda_{ex\text{-}max}$ at or near 470 nm. Although the intensity within each of these fluorescence emission bands varied dramatically across the excitation region from 250 to 650 nm, the wavelength of maximum emission remained within ± 5 nm of the specified location. It was also noted that no new or unique fluorescence features were observed within the measured wavelength region that could be attributed solely to the N regime sampled.

In addition to determining presence and location of fluorescence emission maxima, the EEM allows one to visualize how the fluorescence emission from vegetation responds to changes in excitation wavelength. In Fig. 3, the two emission spectra simulate excitations by the tripled ($\lambda_{ex} = 355$ nm) and doubled frequency ($\lambda_{ex} = 532$ nm) of a Nd:YAG laser. The vertical shaded areas indicate the wavelength and band pass (10-nm FWHM) of the fluorescence emission spectrum that was sampled by the FIS and LIFIS multispectral imaging systems. Noteworthy are the intensity increases from chlorophyll emissions in the red and far-red regions of the spectrum that could be induced if both doubled and tripled Nd:YAG laser frequencies were included in an LIF sensor system.

Table 2
FIS single band response to varying rates of N fertilization for adaxial leaf surfaces obtained from corn at the R3 reproductive stage (Site #1, 1998)

Applied N (kg/ha)	Blue	Green	Red	Far-red
244	226 a*	94 a	87 a	96 a
203	196 b	89 ab	75 ab	85 ab
162	190 b	77 cd	70 bc	82 b
122	185 b	78 cd	75 ab	85 ab
81	186 b	82 bc	66 bcd	77 bc
41	191 b	81 bc	55 d	66 c
20	194 b	86 abc	56 cd	69 c
0	153 c	70 d	35 e	51 d

Means ($n = 12$) are based on four replicates with three sub-samples each.

*Means with the same letter are not significantly different by $LSD_{0.05}$ comparisons within fluorescence band.

Table 3
A comparison of linear correlation coefficients (r, $n = 32$) between adaxial FIS image intensities and the following physiological measures of plant productivity: total chlorophyll concentration, leaf N/C, LAI, and grain yield (Site #1, 1998)

Fluorescence band	Growth stage	Total chlorophyll	N/C	LAI	Yield
Blue	V12	0.62**	0.47**	0.72**	0.36*
	VT	ns	ns	ns	ns
	R3	0.41*	0.49**	0.69**	0.48*
Green	V12	ns	ns	ns	ns
	VT	ns	ns	ns	ns
	R3	ns	ns	0.78**	ns
Red	V12	0.81**	0.81**	0.84**	0.72**
	VT	0.53**	0.40*	0.49**	0.47**
	R3	0.84**	0.83**	0.87**	0.90**
Far-red	V12	0.79**	0.78**	0.82**	0.70**
	VT	0.42*	ns	ns	ns
	R3	0.86**	0.85**	0.87**	0.90**

* Strength of association t-test probability coefficient ($0.01 \le p \le 0.05$).
** Strength of association t-test probability coefficient ($p < 0.01$).

3.3. Fluorescence characterization of N fertilization level

Leaf fluorescence emissions, obtained with FIS, significantly increased with N fertilization, reached a plateau for optimal rates, and declined slightly at high N rates (Table 2). The greatest changes for the single band analysis occurred at the R3 stage in the red and far-red bands. Similar trends were observed at the vegetative and tasseling growth stages.

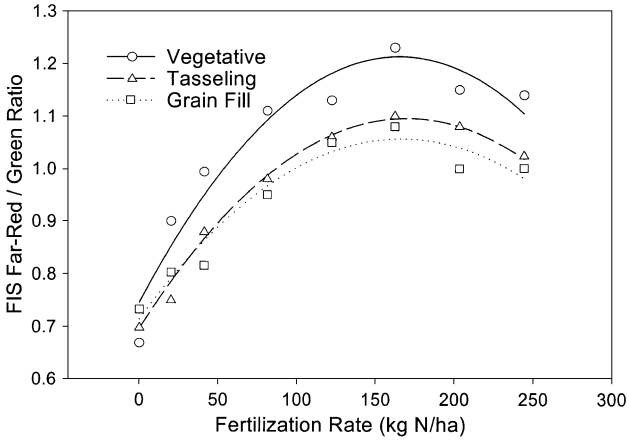

Fig. 4. Response of the far-red/green fluorescence imaging ratio to varying rates of N fertilization (Site 1, 1998). Each symbol represent a treatment mean ($n = 12$) while each line was generated by a quadratic regression analysis ($n = 96$). The far-red/green curvilinear response as a function of growth stage is described by the following equations: V12, $y = 0.746 + 5.67e^{-3}x - 1.72e^{-5}x^2$ ($r^2 = 0.70$, $S_e = 0.1111$); VT, $y = 0.697 + 4.71e^{-3}x - 1.39e^{-5}x^2$ ($r^2 = 0.81$, $S_e = 0.0710$); R3, $y = 0.714 + 4.11e^{-3}x - 1.24e^{-5}x^2$ ($r^2 = 0.73$, $S_e = 0.0804$).

Table 4
FIS leaf and LIFIS canopy fluorescence band ratio response to varying rates of N fertilization obtained from corn at the R3 reproductive stage (Site #1)

Applied N (kg /ha)	FIS 1998 leaf level			LIFIS 2000 canopy level		
	Blue/ Green	Red/ Green	Red/ Far-red	Blue/ Green	Red/ Green	Red/ Far-red
244	2.38 ab*	0.92 ab	0.90 a	1.60 a	0.50 a	0.84 ab
203	2.20 bc	0.84 ab	0.88 ab	1.52 ab	0.47 ab	0.84 ab
162	2.45 a	0.88 ab	0.84 bc	1.42 bc	0.46 ab	0.87 a
122	2.38 ab	0.95 a	0.87 abc	1.40 bc	0.45 b	0.82 b
81	2.28 abc	0.81 b	0.85 abc	1.30 cd	0.41 b	0.76 c
41	2.34 abc	0.67 c	0.81 c	1.26 d	0.38 c	0.81 b
20	2.27 abc	0.66 c	0.82 bc	1.27 d	0.37 c	0.77 c
0	2.19 c	0.51 d	0.69 d	1.19 d	0.36 c	0.75 c

* Means ($n = 12$) with the same letter are not significantly different by $LSD_{0.05}$ within column comparisons.

Significant correlations were noted among emission band intensities from adaxial leaf surfaces and measures of plant productivity during the three growth stages (Table 3). The highest correlations occurred with the far-red band at the R3 stage, for grain yield ($r = 0.90$), LAI ($r = 0.87$), total chlorophyll concentration ($r = 0.86$), and N/C ($r = 0.85$). Similar correlations were also noted for the red band (Table 3).

Of the possible combinations for two band FIS ratios, the blue/green, red/far-red, red/blue, red/green, far-red/blue, and far-red/green all demonstrated similar nonlinear responses to N application rate. This reoccurring nonlinear response is represented by the far-red/green ratio shown in Fig. 4 where maximum ratio values were achieved at or near optimal fertilization while N over-supply led to subtle decreases and N shortage leads to more severe decreases in ratio values. The imaging ratios appeared more sensitive to N deficiency than to over-fertilized N rates (Table 4), but were more sensitive to N levels overall, as compared to single bands. Most importantly, the trends in fluorescence image ratios were consistent across the FIS leaf level and LIFIS canopy level measurements (Table 4).

The similarity in biophysical responses (Figs. 1 and 4) with respect to N fertilization rate lead to a number of significant linear correlations among FIS image ratios and measures of plant productivity (Table 5). High degrees of association were achieved at the R3 reproductive stage with the far-red/green imaging ratio vs. chlorophyll content ($r = 0.98$), grain yield ($r = 0.95$), and leaf N/C ($r = 0.93$).

Table 5
A comparison of linear correlation coefficients (r, $n = 32$) of adaxial FIS band ratios to the following physiological measures of plant productivity; total chlorophyll concentration, N/C, LAI, and grain yield

Band ratio	Growth stage	Total chlorophyll	N/C	LAI	Yield
Blue/Green	V12	0.55**	0.50**	0.52**	ns
	VT	0.43*	0.56*	0.52**	0.51**
	R3	ns	ns	ns	0.60*
Red/Far-red	V12	0.79**	0.85**	0.73**	0.75**
	VT	0.49**	ns	0.63**	0.44*
	R3	0.53*	ns	0.71*	0.69**
Red/Blue	V12	0.75**	0.82**	0.71**	0.71**
	VT	0.73**	0.65**	0.74**	0.62**
	R3	0.93**	0.89**	0.80*	0.92**
Red/Green	V12	0.89**	0.88**	0.86**	0.72**
	VT	0.77**	0.83**	0.87**	0.75**
	R3	0.96**	0.94**	0.82*	0.92**
Far-red/Blue	V12	0.69**	0.75**	0.66**	0.64**
	VT	0.75**	0.67**	0.71**	0.63**
	R3	0.66*	0.64*	ns	ns
Far-red/Green	V12	0.89**	0.87**	0.86**	0.70**
	VT	0.81**	0.87**	0.88**	0.79**
	R3	0.98**	0.93*	0.78*	0.95**

* Strength of association t-test probability coefficient ($0.01 \le p \le 0.05$).
** Strength of association t-test probability coefficient ($p < 0.01$).

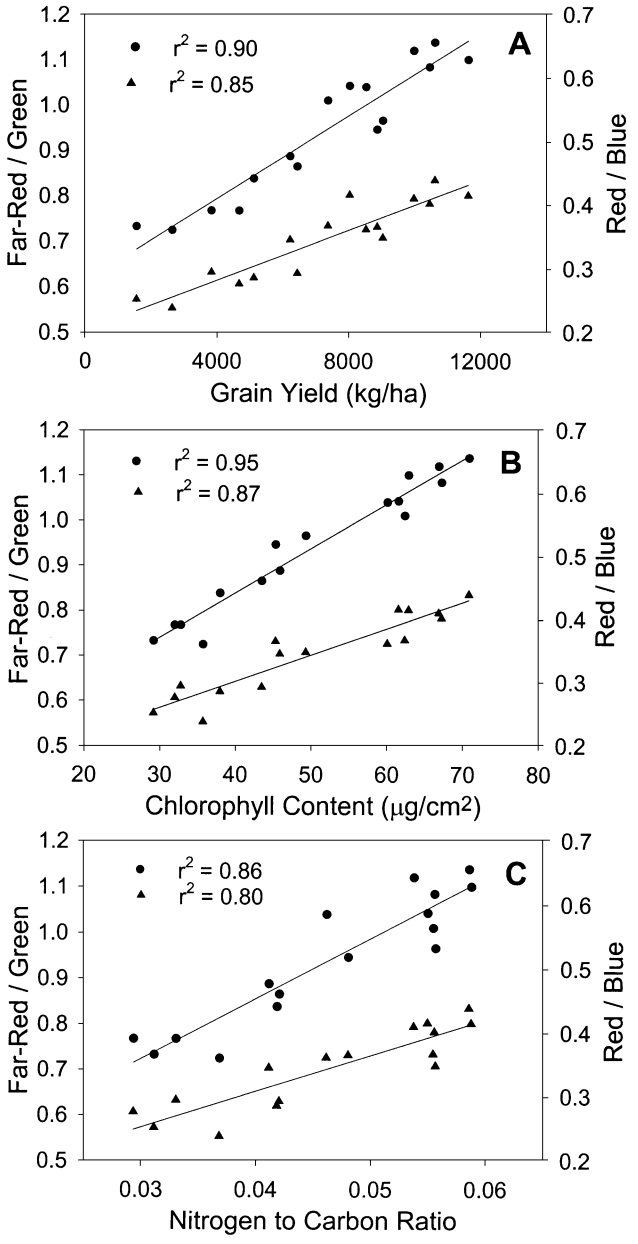

Fig. 5. Linear relationships for the far-red/green (circles) and red/blue (triangles) adaxial leaf surface FIS band ratios (R3) and site-specific grain yield (A), chlorophyll content (B), and leaf N/C ratio (C).

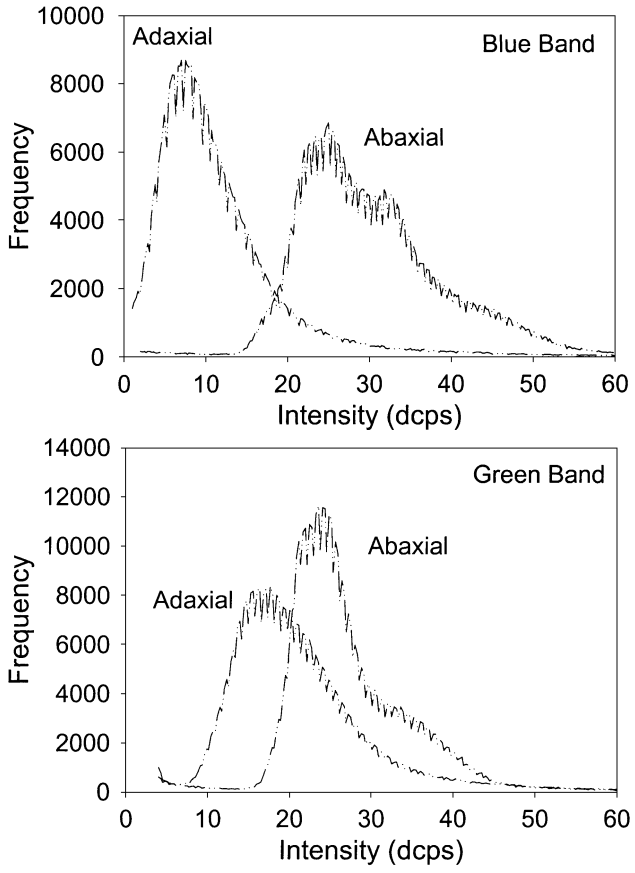

Fig. 6. Distributions of pixel intensity in digital counts per second for FIS images in the blue band (top) and green band (bottom). Abaxial and adaxial leaf emissions were acquired on the fourth from terminal leaf on a 140 kg N/ha treatment at the R3 reproductive stage (Site 2, 2001).

Similarly, high correlations were noted for the red/green, and red/blue ratios. The positive linear relationships for the far-red/green and red/blue FIS ratios and corresponding biophysical measures are shown in Fig. 5. Overall, the correlation coefficients were higher and more consistent across growth stages with fluorescence band ratios (Table 5) as compared to the single band analysis (Table 3).

3.4. Adaxial vs. abaxial fluorescence characterization

Patterns in abaxial vs. adaxial fluorescence emissions were characterized from leaves grown at field Site #2 in 2001. Adaxial single band emissions were not highly

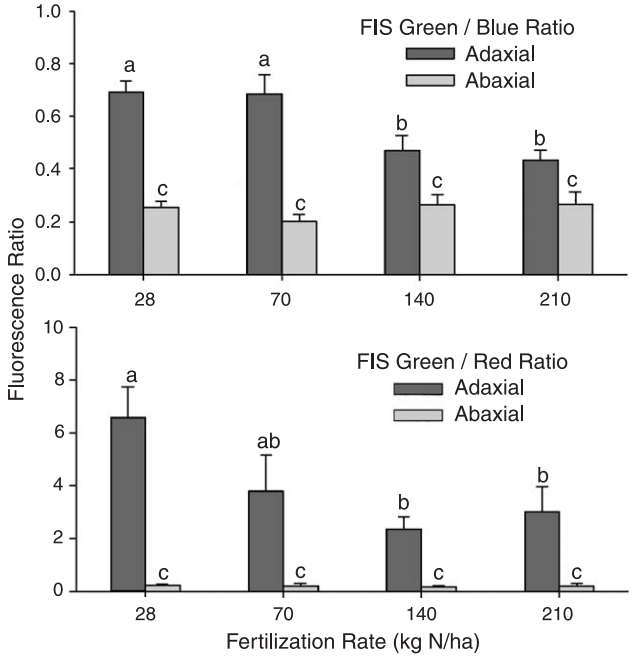

Fig. 7. Green/blue and green/red ratios of FIS leaf fluorescence as a function of leaf surface and N fertilization level (Site #2, 2001). Means with the same letter are not significantly different by $LSD_{0.05}$ comparisons within fluorescence band ratio.

associated with abaxial emissions ($r \leq -0.36$). Emissions from abaxial leaf surfaces were higher in intensity and produced distinct frequency distributions in the blue and green bands (Fig. 6), which is indicative of thickened abaxial epidermal cell walls (Mantha, Jonhson, & Day, 2001). Adaxial leaf fluorescence emissions increased with supplied N. A plateau or slight decrease was not observed, indicating that an over supply of N was not achieved at this location with a fertilization rate of 210 kg/ha. Conversely, abaxial blue and green emissions exhibited an inverse relationship to N fertilization level, with emissions decreasing with increasing N application rate (Table 6).

Distinctly different means for the green/blue and green/red FIS band ratios were obtained for abaxial and adaxial leaf surfaces (Fig. 7). For the purposes of distinguishing N-deficient leaf material from the remainder of the plant canopy, the fluorescence ratios can be inverted such that N-deficient adaxial leaf surfaces would be the plant components exhibiting the highest fluorescence ratio intensity. The resulting inverse relationship between N fertilization rate

Table 6
FIS image means ($n = 21$) from adaxial and abaxial leaf surfaces sampled at the grain fill (R3) reproductive stage (Site #2, 2001)

Applied N (kg/ha)	Blue band		Green band		Red band		Far-red band	
	Adaxial	Abaxial	Adaxial	Abaxial	Adaxial	Abaxial	Adaxial	Abaxial
210	225 c*	781 b	82.3 c	142 b	126 c	1460 a	117 b	290 a
140	209 c	699 b	75.5 c	137 b	46.3 cd	1510 a	99.2 bc	260 a
70	156 c	1350 ab	82.6 c	253 b	45.3 cd	1970 a	80.5 c	269 a
28	159 c	1750 a	81.7 c	436 a	17.1 d	2200 a	68.7 c	324 a

* Means with the same letter are not significantly different by $LSD_{0.05}$ comparisons within fluorescence band.

and green/blue and green/red FIS adaxial band ratios may improve the remote sensing capability to distinguish N fertilization rates of field crops in situ.

4. Discussion

The findings presented here are in agreement with several in vivo steady-state fluorescence investigations into N effects on corn. Chappelle, McMurtrey et al. (1984) discovered that blue, red, and red/blue fluoresce emissions decreased under N limited growth conditions. Similar red/blue and far-red/blue responses were also reported by Heisel et al. (1996) and Lichtenthaler and Schweiger (1998). The relationships between fluorescence emissions and N supply are not limited to corn. Corp et al. (1997) observed for soybean (*Glycine max*) that the blue, red, and far-red bands, and the red/blue and far-red/blue band ratios, decreased with N supply. Similar ratio behavior was reported by Heisel et al. (1997) for wheat (*Triticum aestivum*) and Langsdorf et al. (2000) for sugar beet (*Beta vulgaris*). Langsdorf et al. also reported decreased green, blue/green, red/far-red emissions with N supply.

The increased sensitivity of fluorescence band ratios arise from the differential effects that plant stress factors have on each of the classes of compounds responsible for the multiple bands of fluorescence emission (Chappelle, McMurtrey et al., 1984). Fluorescence ratios were selected to describe positive linear relationships to measures of plant growth condition. This study found strong evidence supporting the use of UV-induced blue/green, red/blue, red/green and far-red/green fluorescence ratios in the elucidation of corn N supply. In addition to detecting differences in N supply, these fluorescence ratios successfully tracked the downward shift in the N/C ratio as the crop progressed from vegetative to grain fill. Consequently, select fluorescence ratios (e.g., red/green in Fig. 4) might also be used to monitor N utilization for C sequestration by crops throughout their growth and development.

At plant and canopy sensing levels, fluorescence image ratios can present additional advantages where extraneous factors affect the overall intensity of fluorescence emissions, such as, canopy density, changes in excitation flux density, and range to target. For this study, these factors were held reasonably constant or removed in part by only including portions of the image in the mean calculation whose intensity was above a prescribed threshold. As a result, LIFIS ratios of single band means yielded similar results to FIS leaf level data. Further study will be required to unlock the full potential of LIFIS ratio imagery. For example, changes in canopy architecture could potentially be used to sense stress factors which do not result in a unique change in spectral signature. The leaf inclination angle distribution of corn canopies under optimal growth conditions tends toward horizontal. At view angles of $\pm 45^\circ$ from nadir, the uppermost adaxial leaf surfaces would be the primary fluorescent component contributing to the canopy emission. Under certain forms of stress, the canopy architecture moves from planophile to erectophile, with abaxial leaf surfaces facing outward to minimize light absorption and evapo-transpiration. Under these conditions, one can expect an increased abaxial leaf fraction leading to a stress-induced change in the canopy fluorescence signal.

One limitation of LIFIS, in its current 355-nm frequency tripled Nd:YAG laser configuration, is that it can yield low levels of chlorophyll fluorescence, particularly in certain UV-hardened monocot and tree species. One proposed modification, similar to that of the dual-excitation FLIDAR (Ounis et al., 2001), would be to incorporate the frequency doubled 532-nm Nd:YAG laser excitation to enhance the chlorophyll fluorescence signal and enable a series of dual excitation fluorescence ratios.

5. Conclusions

Current fluorescence technology indicates that there are several regions of the fluorescence emission spectrum which could be utilized to differentiate vegetation grown at varying rates of nitrogen fertilization. The fluorescence response from corn leaves when excited between 350 and 380 nm exhibited a number of significant correlations among single bands and band ratios to measures of plant growth condition, namely, grain yield, LAI, N/C, and chlorophyll contents. Both the fluorescence and physiological measures exhibited similar curvilinear responses to N fertilization level while significant linear correlations were obtained among fluorescence bands and band ratios to measures of plant productivity. By the shape of the response curves and the number of significant correlations to measures of crop productivity, it can be concluded that the fluorescence ratios which contained either a red or a far-red band divided by either a blue or a green band are providing similar information with respect to N supply.

Corn crops are among the highest consumers of N fertilizers in the United States and a rapid quantitative measure of N status for this crop would prove useful to many farming systems where substantial investments are made in the application of N fertilizers. A rapid nondestructive assessment of leaf N would be useful in determining problem spots in fields where organic or chemical supplements may improve soil fertility and crop yield while reducing the potential for contamination of surface and ground waters. The findings from this study indicated that significant relationships exist between N supply and in vivo fluorescence emissions from corn leaves.

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