USE OF NONDESTRUCTIVE SPECTROSCOPY TO ASSESS CHLOROPHYLL AND NITROGEN IN FRESH LEAVES

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Four aspects of factors influencing the accuracy of nondestructive chlorophyll (Chl) and nitrogen (N) measurement in fresh leaves were studied: (1) optimum wavelength (OW) identification; (2) indices development and evaluation; (3) influence of leaf properties; and (4) influence of meter parameters and sampling technique. Results were used to develop indices and prototype meters for Chl and N assessment. Our results indicated that the simple linear coefficient of determination (R^2) between spectral reflectance or transmission and Chl or N in combination with spectral sensitivity was the most reliable method for determining the OW for Chl and N measurement in fresh leaves. There were two ranges of wavelengths, one in visible region (550 - 580 nm) and the other in the red edge region (700 - 730 nm), we determined that had the highest spectral sensitivity and largest R^2 with smallest root mean square error over a wide-range of Chl concentrations (160 – 1188 $\mu mol.m^{-2}$), and could be used as the OW to develop indices for Chl and N assessment. The OW in the red edge region could be used for Chl assessment across all species tested and the OW in the visible region could be used across

anthocyanins-free species. The best indices were the indices developed with the Chl-related OW either from visible or red edge region in combination with a reference wavelength (RW) from the near infrared (NIR) region (750 – 1100 nm) that was sensitive to leaf texture but insensitive to Chl as the form of a simple ratio (R_{RW}/R_{OW}) or normalized difference vegetation index ($R_{RW} - R_{OW}$)/($R_{RW} + R_{OW}$). With RW, the differences in reflectance in the visible and red edge regions caused by variation in leaf texture or other optical properties could be eliminated. This was particularly important when the R^2 of a single-wavelength index was small for Chl or N measurement (e.g. R^2<0.8000 for Chl or R^2<0.6000 for N).

Parameters used by hand-held Chl meters (CCM-200, SPAD-502, and CM-1000) affected their accuracy for Chl and N assessment. Our results showed that SPAD-502 was more accurate than CCM-200 and CM-1000 for assessing Chl and N in fresh leaves. The Chl-sensitive wavelength used by CM-1000 (700 nm) was more accurate for estimating Chl than the wavelengths used by SPAD-502 (650 nm) and CCM-200 (660nm); however, we found that variation in sampling distance, orientation, light intensity, and the inconsistency of light intensity between ambient light sensor and the target leaf made the CM-1000 less accurate than the other two meters. Using the indices and OW determined through our research, we developed three prototype meters that were more accurate than or similar to the commercial hand-held meters in measuring Chl or N in fresh leaves. Among them, the prototype-III was more accurate than all the commercial hand-held meters for Chl and than the CM-1000 for N assessments across all the species we tested.

Use of Nondestructive Spectroscopy to Assess Chlorophyll and Nitrogen in Fresh Leaves

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I understand that my dissertation will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my dissertation to any reader upon request.

Pinghai Ding, Author

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CHAPTER 1

INTRODUCTION

Nitrogen (N), an essential macroelement required for plant growth, is the most commonly used nutrient in fertilizer to increase plant productivity (Below 1995, Meisinger 1984). Excess application of N to crops can lead to contamination of ground and surface water supplies while too little available N can result in reduced yield and profit (Bullock and Anderson 1998). Efficient N management to achieve optimum productivity while preserving and enhancing the crop quality requires frequent plant testing to ensure that neither too much nor too little N is applied. The chlorophylls, Chl a and Chl b, are photosynthetic pigments essential for the conversion of light energy into stored chemical. energy (Evans 1983, Gitelson et al. 2003, Richardson et al. 2002, Seemann et al. 1987, Syvertsen 1987, Uchida et al. 1982; Yoshida and Coronel 1976). Chl concentration in leaves is positively related to leaf N concentration (Costa et al. 2001; Fernández et al. 1994, Filella et al. 1995; Serrano et al. 2000, Taiz and Zeiger, 1998), and is a sensitive indicator of plant stress (Carter and Knapp 2001, Hendry et al. 1987, Peñuelas and Filella 1998). Estimates of Chl concentrations in leaves can therefore be used as an indirect measure of either plant N (Filella et al. 1995, Moran et al. 2000) or plant stress (Carter and Knapp 2001, Hendry et al. 1987, Peñuelas and Filella 1998), or the combination of both. However, both Chl and N are traditionally quantified by time-consuming wet chemical methods in solvent extraction that involve tissue removal from plants (Arnon 1949). More recently, nondestructive optical methods based on light

transmission or reflectance characteristics of leaves have been developed for Chl and N assessment (Adams et al. 1999, Curran et al. 1990, Datt 1999a, Datt 1999b, Gamon and Surfus 1999, Markwell et al. 1995). These nondestructive methods are simple to use, fast, inexpensive, require no chemical analysis, and can be used for intact measurement in the field (Buschmann and Nagel 1993, Gitelson and Merzlyak 1994b, Gitelson et al. 1996a, Gitelson et al. 1996b, Markwell et al. 1995).

Nondestructive assessment of Chl and N by reflectance at a canopy-level using remote sensing or by transmittance at a leaf-level using SPAD-502 and other hand-held meters have been studied extensively over the last 10 years (Gitelson 2002, Markwell et al. 1995). Major advances have been made in understanding (1) interactions between leaf and light characteristics in the visible and infrared regions of the spectrum, (2) how to develop indices for Chl and vegetation (or greenness) assessment, and 3) the effects of leaf properties on the accuracy of leaf Chl and N estimates. However, many aspects that influence the accuracy of Chl and N assessment remain to be elucidated, including (1) methods for selecting and using optimum wavelengths to develop indices for Chl assessment (OW_{Chl}), (2) understanding how the methods for developing indices influence the accuracy of Chl assessment; (3) identifying indices parameters that can be used to increase accuracy of Chl assessment across genotypes; and (4) understanding what factors influence the accuracy of commercially available meters used for Chl and N assessment.

The importance of using OW_{Chl} for indices development is not widely recognized. Many indices have been developed in remote sensing for Chl assessment in numerous plant species (Adams et al. 1999, Blackburn 1998, Curran et al. 1990, Datt 1998, Datt

1999a, Datt 1999b, Gamon & Surfus, 1999, Gitelson and Merzlyak 1994b, 1996; Gitelson et al. 1996a, Gitelson et al. 1996b). These indices, however, can not be used universally across different plant genotypes (species or cultivars). The main reason is that Chl-related wavelengths used to develop indices differ between studies.

The region of rapid increase in reflectance between the red and infrared regions of the spectrum, called the red edge (700 - 730 nm), is frequently used to indicate plant stress and health (Dawson and Curran 1998, Horler et al. 1983a, Horler et al. 1983b, Jago et al. 1999). In fresh leaves, the absorption coefficients of Chl in the blue and red regions of the spectrum are very high (Lichtenthaler 1987) and the depth of light penetration into the leaf is very low (Cui et al. 1991, Fukshansky et al. 1993, Merzlyak and Gitelson 1995). As a result, even a low Chl concentration (e.g. 150$\mu g.m^{-2}$) can sufficiently saturate absorption, and increases in Chl concentration do not result in an increase in total absorption (Gitelson et al. 2003). Chl can absorb more than 80% of incident light from wavelengths in the green (540-590 nm) and red edge (700-730 nm) regions of the spectrum (Gausman and Allen 1973, Gitelson and Merzlyak 1994a). Although the absorption by Chl at these wavelengths is lower than blue and red regions, wavelengths in the green and red edge regions of the spectrum penetrate four- to six-times deeper below the leaf surface than wavelengths in the blue and red region (Fukshansky et al. 1993, Merzlyak and Gitelson 1995). This suggests that absorption of wavelengths in the green or red edge region of the spectrum may result in a high sensitivity of Chl estimates based on reflectance measurements (Gitelson et al. 2003). Commercial hand-held meters for Chl assessment measure transmission of red wavelengths between 620 - 660nm to assess Chl in plant leaves. Theoretically, high light absorption by leaves in combination

with deep light penetration by wavelengths in the green and red edge regions of the spectrum should also result in a high sensitivity of Chl estimates based on transmission measurements; however, there are no reports confirming this hypothesis.

Use of OW_{Chl} and proper indices are very important for increasing the accuracy of nondestructive Chl and N assessment; however, the methods for identifying the OW_{Chl} and selecting proper indices have not been compared and evaluated. The wavelengths and the indices used by canopy-level remote sensing devices and hand-held meters for assessing Chl concentration are generally determined by using either a semi-empirical approach (Aoki et al. 1986; Chapelle et al. 1992, Gitelson and Merzlyak 1996, Lichtenthaler et al. 1996, Yoder and Daley 1990) or a statistical approach (Bolster et al. 1996, Curran et al. 1992, Fukshansky et al. 1993, Gitelson et al. 2003, Grossman et al. 1996, Jacquemoud et al. 1995, Martin and Aber 1994, Merzlyak and Gitelson 1995, Yoder and Pettigrew-Crosby 1995). Using a statistical approach for identifying OW_{Chl} and developing indices is considered more reliable and accurate than using a semi-empirical approach. Statistical methods commonly used include the use of (1) the coefficient determination (R^2) and root mean square error (RSME) from regression of Chl concentrations determined using wet chemistry and reflectance or transmission values, (2) derivatives, and (3) reflectance difference and reflectance sensitivity analyses. Several different methods have been used for Chl-related wavelength selection and indices development; however, the reliability and accuracy of these methods have not been compared.

The effect of leaf properties on indices for Chl or N assessment are well documented (Ahlrichs and Bauer 1982, Andrew et al. 2002, Bullock and Anderson 1998,

Gausman 1974, Schepers et al. 1992, Sunderman and Lamm 1991, Takebe and Yoneyama 1989); however, the influence of plant genotype on indices has not been extensively investigated. It is possible that the variation in indices accuracy for Chl assessment between genotypes is solely a function of genotype variation in the leaf optical properties (leaf thickness, texture, density, Chl content, water status, etc.) that affect indices used for Chl or N assessment.

Hand-held meters have been used extensively for assessing leaf Chl and N in numerous plant species (Bullock and Anderson 1998, Costa et al. 2001, Kantety et al. 1996, Markwell et al. 1995, Nielsen et al. 1995, Richardson et al. 2002, Schepers et al. 1992, Turner and Jund 1991). The accuracy of these meters varies under different measurement conditions because the meter parameters (i.e. meter wavelength, the consistency and constancy of sampling distance and light source, etc.) lack robustness (Jacquemoud and Ustin 2001). The influence of meter parameters on meter accuracy has not been extensively compared and characterized. Richardson et al. (2002) compared the accuracy of two hand-held transmissions Chl meters (SPAD-502 and CCM-200) with reflectance indices developed for canopy-level remote sensing and concluded that relative Chl concentration was more accurately estimated by reflectance than transmission. However, the wavelengths used in their reflectance indices were different from those used in the hand-held meters. Therefore, the differences in the accuracy of Chl assessment between these two hand-held transmission Chl meters and the reflectance indices they developed may have been a result of differences in wavelengths rather than the difference in measuring methods (e.g. reflectance vs. transmittance).

The objectives of this research were to 1) determine the best methods for selecting OW_{Chl} and OW_N and developing indices for Chl and N assessment; 2) characterize how plant genotype and variation in leaf texture, water status, and pigments influence Chl assessment; 3) identify how parameters in hand-held meters used to assess Chl influence meter accuracy; and 4) develop a hand-held meter with higher accuracy and sensitivity for nondestructive Chl and N assessment than commercially available meters.

CHAPTER 2

LITERATURE REVIEW

2.1 Properties of light as electromagnetic radiation

Light is a form of electromagnetic radiation (EMR) and can be classified into γ-rays, X-rays, ultraviolet radiation, visible light, infrared radiation (near infrared, middle infrared, far infrared), microwaves and radio waves (Figure 2.1). Each wavelength of light is associated with a specific photon, or particle of energy (Bokobza 1998, Current 1989, Murray and Williams 1987). In general, shorter wavelengths have higher frequencies and more energy than longer ones. The interaction of solar radiation with molecules in plant leaves not only controls plant photosynthesis and other important metabolic processes, it is also the basic principle used for spectroscopic assessment of Chl and other molecules. Molecules can absorb photons of energy if the photons have energy coincident with the characteristic vibrations of the molecule.

The fundamental absorption wavebands with the most intense absorption of energy in leaves occur at wavelengths between 280 - 2800 nm. In general, the most important optical range of wavebands for nondestructive measurement of molecules in leaves ranges from 400 - 2500 nm and is divided into four regions: visible (400 - 700 nm), red edge (700 - 750 nm), near infrared (NIR, 750 - 1300 nm) and middle infrared (MIR, 1300 - 2500 nm) (Figure 2.2, Jacquemoud and Ustin, 2001). The red edge (700 - 750 nm) is the region between the red and infrared regions of the spectrum (Richardson and Berlyn 2002, Dawson and Curran 1998). Many researchers classify red edge wavebands as NIR region wavelengths between 700 - 1300 nm (Dawson and Curran 1998, Horler et al. 1983a).

2.2 Interactions between leaves and visible, red edge and infrared radiation

2.2.1 Influence of wavelength on interactions between light and leaves

The fundamental theory of light-matter interaction is Maxwell's electromagnetic wave theory (Fukshansky et al. 1993). Light, widely thought to move through leaf cells and tissues as rays, is actually wave-like. Ray motion is a special simple case of wave motion (Latimer 1984). Biochemical and structural components in plants influence their ability to absorb, transmit, and reflect different wavelengths. EMR absorption by plants is controlled by molecular interactions within plant tissues, where the electrons in molecules absorb incoming solar radiation at wavelengths specific to chemical bonds and structure (Gates 1980, Jones 1997). Therefore, changes in the concentrations of absorptive molecules cause changes in leaf absorbance, transmittance, and reflectance.

The visible region of the spectrum is characterized by a strong absorption of light by photosynthetic pigments in a green leaf. Absorption of NIR region wavelengths is limited to dry matter and related to the proportion of the leaf composed of air spaces, i.e., the internal structure of the leaf affects the amount of light reflectance and transmittance. Absorption of red edge region wavelengths by Chl pigments is low and reflectance is high. Changes in reflectance of red edge wavelengths are often associated with Chl concentration (Moran et al. 2000) and are used as an indicator of plant stresses and health (Dawson and Curran 1998, Horler et al. 1983a, Horler et al. 1983b, Jago et al. 1999). This is why the reflectance of red edge wavebands is more commonly used than visible light for detecting vegetation or greenness differences among plant species in remote sensing (Moran et al. 2000, Richardson et al. 2002). The peak reflectance of intact leaves is in the NIR region. Changes in NIR reflectance are primarily caused by changes in plant

structure (Merzlyak et al. 2003). Reflectance of wavelengths from the NIR region is thought to be controlled by the complex nature of the cavities within the leaf and internal reflectance of infrared radiation within these cavities (Jacquemoud and Ustin, 2001). Wavelengths from the MIR region are also strongly absorbed by leaves; primarily by water in fresh leaves, but also by dry matter when the leaf wilts (Jacquemoud and Ustin 2001).

The spectral characteristics of a leaf changes as it matures or experiences stress. For example, stress may cause reduction in Chl, which leads to changes in absorption of blue and red light and an increase in overall reflectance of wavelengths from the visible region of the spectrum. Changes in red edge and NIR reflectance during periods of stress are often more noticeable than changes in the visible region (Gamon et al. 1992). Because of variations in leaf pigment concentrations, leaf water content, and leaf structure, the leaves of different vegetation types differ in terms of how they interact with EMR. As plants mature or are subjected to stress by disease, insect attack, or moisture shortage, the spectral characteristics of leaves may change (Figure 2.2). In general, these changes apparently occur more or less simultaneously for wavelengths from the visible, red edge and NIR regions, but changes in NIR reflectance are often more noticeable.

2.2.2 Influence of leaf anatomical structure on interactions between light and leaves

The interactions between leaves and EMR are a function of leaf anatomical structure. In cross section, a typical leaf from adaxial to abaxial surface consists of the upper cuticle and epidermis, palisade tissue, spongy mesophyll tissue, and the lower epidermis and cuticle (Figure 2.3). The cuticle and epidermal cell layer diffuse and transmit most of the incident

light. The palisade layer contains chloroplasts, which hold Chl pigments. Chl absorbs most visible light (up to 70-90% of blue, red, and green wavelengths). However, more of the green light that comes into contact with leaves is reflected than blue and red light, causing green to be the prominent color of leaves according to the human eye. The absorbance peak of Chl in the blue region of the spectrum overlaps with the absorbance of carotenoids, so blue reflectance is not generally used to estimate Chl concentration (Sims and Gamon, 2002). The maximum absorbance in the red region of the spectrum occurs between 660 - 680 nm (Curran, 1989), but relatively low Chl concentrations can saturate absorption in this region (Sims and Gamon, 2002).

Chl absorption is primarily influenced by electron transitions between 430 - 460 nm and 640 - 660 nm (Curran, 1989; Taiz and Zeiger, 1998). The spongy mesophyll tissue in leaves regulates the leaf interaction with wavelengths from the NIR region of the spectrum. The cuticle and epidermis are almost completely transparent to NIR wavelengths, so very little NIR radiation is reflected from the outer portion of the leaf. NIR radiation passing through the upper epidermis is strongly scattered by mesophyll tissue and cavities within the leaf. Very little of this NIR radiation is absorbed internally, most (up to 60%) is scattered (reflected) upward or transmitted downward (Campbell 1996). Thus the internal structure of the leaf is responsible for the reflectance or transmission of wavelengths from the NIR region.

Mesophyll layers with a high proportion of air spaces between cells reflect more light in the NIR than leaves with more compact or dense mesophyll layers. There are significant structural differences in the mesophyll layers between plants, causing them to reflect varying amounts of light from the NIR region of the spectrum. Mesophyll cells and air spaces

strongly reflect and transmit incoming radiation. Reflectivity in the NIR varies more between species than reflectivity in the visible region of the spectrum, allowing people to efficiently classify healthy vegetation using NIR light. The interactions between leaves and visible and NIR radiation have been described using a stochastic radioactive transfer model (Figure 2.4). In this model the leaf is partitioned into different tissues. Light reflectance, transmission and absorption occur at each layer of tissue like a Markov chain (Tucker and Garatt 1977, Maier et al. 1999). The internal leaf structure and the optical constants of the leaf tissue control the interaction between the leaf and EMR (Allen et al. 1973, Brakke and Smith 1987, Kumar and Silva 1973, Govaerts et al. 1996, Baranoski and Rokne 1997, Ustin et al. 2001).

The properties of light and the interactions between leaves and visible, red edge and NIR radiation are the basic theories used to develop instrumentation for assessing plant Chl, N, and stresses based on leaf optical properties.

2.3 Measuring plant leaf and EMR interactions

2.3.1 Definitions of Reflectance, Transmittance, and Absorbance

Reflectance and transmittance are defined as the ratios of reflected or transmitted radiation to incident radiation. Incident radiation that is not reflected or transmitted by a leaf is presumed to be absorbed. Reflectance and transmittance are presented as either a percent or as a fraction of incident radiation. Absorption is characterized either as a ratio of incident radiation or as a function of optical density (Porra et al. 1989, Rabideau et al. 1946).

2.3.2 Instrumentation

Instruments that measure quantities of visible and NIR radiation based either on reflectance or transmittance use detectors made from photoexcitable materials such as silicon or indium gallium arsenide (InGaAs). Silicon is a common photoexcitable material that produces an electrical current in response to visible and most of the NIR radiation (300 - 1100 nm). However, silicon does not respond to radiation above 1100 nm, so more expensive materials, such as InGaAs detectors, are used for measuring wavelength above 1100 nm in both NIR (1100 - 1300 nm) and MIR (1300-2500 nm). Most published research on nondestructive assessments of Chl or N has either focused on canopy-level reflectance measurements for remote sensing (Best and Harlan 1985, Curran 1989, Curran et al. 2001, Carter and Spiering 2002, Dawson 2000, Demetriades-Shah et al.1990, Dusek et al. 1985, Fernández et al. 1994, Gao 1996, Huete et al. 1985, Kokaly and Clark 1999, Major et al. 1990, Sims and Gamon 2002, Peñuelas et al. 1994, Peñuelas et al. 1985, Peñuelas et al. 1997, Tian et al. 2001) or leaf-level transmission measurements at two wavelengths using hand-held meters (Bullock and Anderson 1998, Carter and Spiering 2002, Costa 2001, Kantety et al. 1996, Monje and Bugbee 1992, Nielsen et al. 1995, Richardson et al. 2002, Schepers et al. 1992, Markwell et al. 1995, Turner and Jund. 1991).

2.3.2.1 Hand-held meters for Chl assessment

Output from hand-held meters used for Chl assessment, including SPAD-502 (Minolta Corp., Japan), CCM-200 Chl Content Meter (Opti-Science, Inc., Tyngsboro, MA), CL-01 Chl content meter (Hansatech Instruments, England) and CM-1000 Chl Meter (Spectrum Technologies, Inc., Plainfield, IL), is positively correlated with leaf Chl and N concentrations in leaves of many annual, perennial, and woody plant species

(Bullock and Anderson 1998, Costa 2001, Kantety et al. 1996, Nielsen et al. 1995, Richardson et al. 2002, Schepers et al. 1992, Markwell et al. 1995, Turner and Jund. 1991). The accuracy of estimates differs among these hand-held meters even though estimates from all meters are based on leaf response to light at two wavelengths: one Chl-sensitive wavelength and one Chl-insensitive wavelength (Markwell et al. 1995, Minolta 1989, Opti-Science 2000, Whaley 2001).

The CCM-200 weighs 180 g, has a 0.71cm^2 measurement area, and calculates a Chl content index (CCI) based on absorbance measurements at 660 nm and 940 nm. The claimed accuracy of the CCM-200 is ±1.0 CCI units. The SPAD-502 weighs 225 g, has a 0.06 cm^2 measurement area, and calculates an index in SPAD units based on absorbance at 650 nm and 940 nm. The claimed accuracy of the SPAD-502 is ±1.0 SPAD units. CL-01 Chl content meter weighs 250g, can measure leaf samples up to a maximum of 12.7cm wide, and calculates a Chl index based absorbance at 620 nm and 940nm. The CM-1000 weighs 692g and calculates an index in CM-1000 units based on reflectance at 700 nm and 840 nm. The recommended sampling distance for the CM-1000 is 28.4 - 183.0 cm with a corresponding sampling scope of 1.10 - 18.8 cm in diameter outlined with the high powered lasers.

The hand-held transmission meters (SPAD-502, CCM-200 and CL-01) use two light emitting diodes (LEDs) to produce red light with peaks of 620 nm (CL-01), 650 nm (SPAD-502) or 660 nm (CCM-200) and NIR light with a peak of 940 nm. The functions of the red and the NIR wavelengths are different. Leaf absorbance and transmission of the red wavelength are sensitive to changes in leaf Chl concentrations, whereas that of the NIR wavelength are sensitive to leaf texture (Markwell et al. 1995). Therefore the 620

nm, 650 nm or 660 nm wavelengths are used to measure leaf Chl while the 940 nm wavelength serves to compensate for leaf texture differences such as tissue thickness (Minolta 1989; OptiScience, 2000).

In 80% acetone, Chl a and Chl b can be measured by using the red wavelengths 663.2 nm and 646.8 nm, respectively. Total Chl concentration is derived from the sum of Chl a and Chl b (Lichtenthaler and Wellburn 1983). Most Chl in plant leaves is in the form of Chl a, thus, total Chl in extracted solution can also be directly measured by using 650 nm and 660 nm wavelengths in the red region (Lichtenthaler 1987). This is possibly the reason that SPAD-502 and CCM-200 use a 650 nm and 660 nm wavelength, respectively, to assess Chl in plant leaves. However, in fresh leaves the optimal wavelength for Chl assessment (OW_{Chl}) is very different from that of acetone extracts of Chl from leaves (Gitelson et al. 2003). In fresh leaves, the absorption coefficients of Chl in red region of the spectrum are very high (Lichtenthaler 1987) and the depth of light penetration into the leaf is very low (Cui et al. 1991, Fukshansky et al. 1993, Merzlyak and Gitelson 1995). As a result, even leaves containing low concentrations of Chl can saturate absorption of wavelengths in the red region of the spectrum. When Chl exceeds $150 \mu g.m^{-2}$, total absorption reaches a maximum, and an increase in Chl concentration does not cause an increase in absorption (Gitelson et al. 2003).

Specific absorption coefficients of wavelengths from green and red edge regions of the spectrum by Chl extracts (i.e. 80% acetone) are very low and less than 6% of the absorption coefficients of wavelengths in the blue and red regions (Heath 1969, Lichtenthaler 1987). However, fresh green leaves absorb more than 80% of incident light from wavelengths in the green and red edge regions (Gausman and Allen 1973, Gitelson

and Merzlyak 1994a). In these spectral regions, depth of light penetration into the leaf can be four- to six-fold higher than light from wavelengths in the blue and red regions (Fukshansky et al. 1993, Merzlyak and Gitelson 1995). Therefore, absorption of light from the green and red edge regions is great enough to provide a high sensitivity for using reflectance to assess Chl (Gitelson et al. 2003). Theoretically, absorption of light by leaves is not affected by the measuring method of either reflectance or transmission. The reflectance of wavelengths from both the green and red edge regions is sensitive to Chl concentration in leaves, therefore transmission of wavelengths from these regions should also have a high sensitivity when used for Chl assessment; however, no commercially available hand-held transmission meters use these wavelengths from these regions for assessing Chl.

2.3.2.2 Multiple-wavelength spectroradiometery

Canopy-level remote sensing uses both narrowband and broadband spectroradiometers to assess greenness or provide a relative vegetation index. Narrowband spectroradiometers are commonly used for ground-based and aerial imaging platforms, while broadband spectroradiometers are generally used in satellites with spatial imaging capabilities sufficient to measure cropland. Narrowband spectral indices are used to measure slope (Demetriades-Shah et al. 1990, Peñuelas et al. 1994), shape (Tian et al. 2001), and depth (Curran et al. 2001, Kokaly and Clark 1999) of absorption bands, while broadband indices only measure the depth.

Compared to hand-held meters, which use two wavelengths and yield a single index value for estimating Chl, portable spectroradiometers measure both reflectance and

transmittance across the entire spectrum from ultraviolet, visible to NIR wavelengths (Curran et al.1990, Adams et al. 1999, Datt 1999, Gamon & Surfus 1999, Schepers et al. 1996). Thus, by analyzing the entire spectrum, researchers can obtain an almost infinite number of indices and more useful information (Richardson et al. 2002). Use of multiple-wavelength analyses also improves researchers' ability to compare, choose and evaluate OW_{Chl} and indices for assessment of Chl and other pigments (Adams et al. 1999, Lichtenthaler et al. 1966, Merzlyak et al. 2003). However, choosing an appropriate transformation index from the vast array of derived indices is problematic (Richardson et al. 2002) and there is no widely accepted method for doing so.

2.3.3 Spectral indices

2.3.3.1 Indices used in the hand-held meters

Measurements with SPAD-502, CCM-200 and CL-01 are all based on a ratio of leaf transmission of light at two wavelengths, while CM-1000 is based on the ratio of leaf reflectance of light at two wavelengths. The algorithm used in SPAD-502 for the ratio calculation appears to be different from that of CCM-200, CL-01 and CM-1000. The ratio for SPAD-502 is based upon initial calibration measurements obtained by closing the sampling head without leaf sample. During this calibration procedure the built-in microprocessor receives photodiode voltages of V_{650} and V_{940} produced by the red (650nm) and NIR (940nm) light beams and stores the digital values in memory. When a leaf is subsequently measured, the microprocessor receives the voltages of V'_{650} and V'_{940} produced by the red and NIR lights transmitted through the leaf, and the SPAD-502 reading or output is an index based on the ratio of the voltage produced by each

wavelength to the corresponding values stored in the memory. The SPAD-502 reading can be calculated by the Eq. (1), in which the transmission related wavelength voltage replaces the current used by Markwell et al. (1995).

$$SPAD-502\ output = Log\frac{V'_{940}/V_{940}}{V'_{650}/V_{650}} = Log\frac{V'_{940}\cdot V_{650}}{V'_{650}\cdot V_{940}} \quad (1)$$

Using this algorithm, a leaf with a higher Chl concentration will absorb more light than a leaf with a lower Chl concentration; therefore, less light will be transmitted through the leaf and sensed by the photodiode. Less light received by the photodiode results in a lower voltage V'_{650} and the meter will generate a larger reading. Conversely, a leaf with lower Chl concentration will absorb less light and transmit more light to the photodiode, resulting in a higher V'_{650} and a smaller reading.

CM-1000 uses external light (e.g. ambient) at 700 nm and 840 nm wavelengths to estimate the quantity of Chl in leaves (Whaley 2001). Chl absorbs the 700 nm light and, as a result, the reflection of light at that wavelength from the leaf is reduced compared to the reflected light at the 840 nm wavelength. Light having a wavelength of 840 nm is unaffected by leaf Chl concentration and serves as a parameter to compensate for leaf structural differences such as the presence of a waxy or hairy leaf surface. The quantity of ambient light (840 nmA and 700 nmA) and the sample reflected light (800 nmS and 700 nmS) at each wavelength is measured and converted into corresponding voltage (V_{840A}, V_{700A}, V_{840S} and V_{700S}). The output index is calculated from Eq. (2). Similar to SPAD-502, a leaf with a higher Chl concentration will absorb more light than a leaf with a lower Chl concentration; therefore, less light will be reflected by the leaf and sensed by the photodiode, resulting in a smaller V_{700S} and a larger reading. Conversely, a leaf with

lower Chl concentration will absorb less light and reflect more light to the photodiode, resulting in a higher V_{700S} and a smaller reading.

$$CM - 1000\,output = (V_{840S}/V_{840A})/(V_{700S}/V_{700A}) \quad (2)$$

Meter parameters (i.e. meter wavelength, sampling distance uniformity and light source, etc) influence the meter accuracy. Among these parameters, the Chl-related wavelength is the most important parameter determining meter accuracy; however, all the Chl-related wavelengths used by hand-held meters are not the OW_{Chl}. Moreover, the algorithms used by hand-held meters are based on the assumption that Chl is uniformly distributed within the leaf and light intensity within the leaf is uniform. These assumptions either ignore scatterance, reflectance, and Chl fluorescence when measuring transmission or assume that light transmittance, absorptance, scatterance, reflectance, and Chl fluorescence are all proportional to leaf Chl concentration. However, like most biological materials, plant leaves are not perfect optical systems (Vogelmann 1993). Chl pigments are localized within chloroplasts, which are not uniformly distributed within leaves, and light may pass through microenviroments with different Chl concentrations (Markwell et al. 1995). Chl fluorescence contributes 1 - 3% of the light absorbed by Chl (Nobel 1991), whereas individual contributions of absorptance, scatterance and reflectance are difficult to access because the relationships among them are very complex (McClendon and Fukshansky 1990, Vogelmann 1993). If significant amount of scatterance and reflectance occur, and their value cannot be estimated, they may simultaneously decrease the transmission through the leaf (McClendon and Fukshansky 1990) and lead to an overestimation of Chl concentration (Markwell et al. 1995).

2.3.3.2 Indices used in vegetation evaluation by remote sensing

Indices are the key parameters used in nondestructive spectral assessment of Chl and N in leaves. An abundance of indices (Table 2.1) are available for Chl and N assessment or characterization of vegetation by remote sensing (Elvidge and Chen 1995, Jackson 1983). Almost all these indices are developed based on reflectance at either canopy- or leaf-level by using either a single Chl-related wavelength (i.e. 550, 698, 692 or 695 nm) (Thomas and Gausman 1977, Jacquemoud and Baret 1990, Cater 1994, Cater 1998, Moran and Moran 1998) or a Chl-related wavelength with a Chl-insensitive wavelength. The most popular indices used in remote sensing are developed with more than one wavelength, including: (1) simple ratio (SR), (2) normalized difference vegetation index (NDVI), (3) photochemical reflectance index (PRI), (4) structure independent pigment index (SIPI) (5) red edge position (λRE), (6) first-order derivative green vegetation index (FDGVI; Elvidge and Chen 1995), or (7) reflectance integral index (RII) (Gitelson & Merzlyak 1994b, Richardson et al. 2002).

A SR is one of the most frequently used indices in remote sensing to assess the abundance and vigor of vegetation and is calculated as the ratio of reflectance values of two single wavelengths. The SR is also called vegetation index (VI) if the ratio is between wavelengths from the NIR region and the red region (e.g. $VI=R_{NIR}/R_{Red}$; where R_{NIR} is the reflectance value in NIR and R_{Red} is the reflectance value in red region of the spectrum) (Richardson *et al.* 2002, Jordan 1969). Some indices are developed specifically for either Chl a or Chl b, therefore the SR is called a pigment specific SR for Chl a (PSSR a) and Chl b (PSSR b) (Blackburn 1998).

A NDVI is also commonly used in remote sensing (Gamon and Qiu 1999) to deal with variations of topography and illumination and positively correlated with leaf Chl concentration (Peñuelas & Filella 1998, Richardson et al. 2002). A NDVI is calculated as the proportion of the difference in reflectance values of two single wavelengths to the sum of reflectance values of the two wavelengths [e.g. $(R_{NIR}-R_{Red})/(R_{NIR}+R_{Red})$]. A frequently used NDVI is calculated as NDVI = $(R_{750} - R_{675})/(R_{750} + R_{675})$. A modified version of the NDVI, the Chl Normalized difference index (Chl NDI), has a higher correlation with leaf Chl concentration and is more sensitive to a wider range of Chl concentrations. The Chl NDI calculated as Chl NDI = $(R_{750} - R_{705})/(R_{750} + R_{705})$ (Gitelson and Merzlyak 1994b, Richardson et al. 2002).

A PRI is an index of xanthophyll cycle pigment activity (Gamon and Surfus 1999, Peñuelas and Filella 1998) and is frequently used for measuring photosynthesis efficiency. Over short time spans (e.g., diurnally), PRI is correlated with both the epoxidation state of xanthophyll cycle pigments and photosynthetic radiation use efficiency (PRUE; PRUE = [(net photosynthesis) / (incident photosynthetically active radiation)]) (Gamon et al. 1992, Peñuelas et al. 1995b, Filella et al. 1995). Over longer time spans, or across species or sites, PRI is positively correlated with photosystem II (PSII) efficiency as measured by Chl fluorescence and the ratio of Chl:carotenoids, which may itself be an indicator of PSII efficiency (Sims and Gamon 2002).

An SIPI is an index associated with the ratio of total carotenoids (reflectance at 445 nm) to Chl a (reflectance at 680 nm) [e.g. $(R_{800}-R_{445})/(R_{800}-R_{680})$] (Moran et al. 2000, Peñuelas et al.1995b). SIPI is used in remote sensing for detecting plant greenness (Moran et al. 2000, Peñuelas et al.1995b).

The red edge position (λRE) (Current et al. 1990) is the wavelength (λ, nm) with the greatest slope in the reflectance spectrum between 690 nm and 740nm. A λRE is frequently used in remote sensing for detecting various plant related stresses and determined from the maximum of the first-difference (1st derivative) spectrum. A λRE is calculated as $(R_n-R_{n-1})/(\lambda_n-\lambda_{n-1})$; where R_n is reflectance at wavelength n and λ_n is the wavelength n. The 1st derivative spectrum measures change in reflectance from one wavelength to the next and is a measure of the slope of the raw reflectance spectrum (Richardson et al. 2002).

The FDGVI is used in remote sensing for estimating greenness and is calculated from the slope of the raw reflectance spectrum at different wavelengths. The FDGVI measures the change in reflectance from one wavelength to the next (Eq. 3) (Richardson et al. 2002).

$$\text{FDGVI} = \sum_{\lambda_1}^{\lambda_n} \rho(\lambda_i) / \Delta\lambda_j \qquad (3)$$

A RII is an index used in remote sensing that is related to 'greeness'. This index is calculated using a discrete summation approximation to the integral (Eq. 4) (Gitelson and Merzlyak 1994a).

$$\text{RII} = \int_{705}^{750} (R_\lambda / R_{705} - 1)\text{d}\lambda \qquad (4)$$

2.3.4 Factors affecting Chl and N assessment

Numerous researchers have described how variation in leaf spectral properties is related to leaf biochemical composition and structure differences that are a result of many factors affecting Chl and N assessments in canopy or leaves (e.g. species, developmental

stage, microclimate, position on the plant, abiotic and biotic stresses, etc.). Richardson et al. (2002) concluded that differences in leaf structure and the associated effects on reflectance severely impair our ability to use many indices across a wide range of vegetation types. In addition, such differences make it unlikely that index or calibration equation from one study can be directly applied to leaves with different structural attributes (Richardson et al. 2002). This is why many indices developed for nondestructive assessment of Chl (Curran et al. 1990, Gitelson & Merzlyak 1994b, Gitelson & Merzlyak 1996b, Gitelson et al. 1996a, Gitelson et al. 1996b, Blackburn 1998, Datt 1998, Datt 1999b, Adams et al. 1999, Gamon and Surfus 1999) can not be used across a wide variety of plant species.

2.3.4.1 Plant species

The influence of species or genotype on leaf reflectance has been characterized by several researchers (Bullock and Anderson 1998, Peng et al. 1993, Schepers et al. 1992, Sunderman and Lamm 1991, Takebe and Yoneyama 1989). A leaf is composed of layers of structural organic matter, within which are pigmented, water-filled cells and air spaces. These three features – pigmentation, anatomical structure, and water content, have an effect on the reflectance, absorbance and transmittance properties of leaves (Current 1985). The structure of the cuticle and epidermal layer(s) control diffusion and transmission of the incident light, whereas the palisade layer and spongy mesophyll tissue affect the interactions between leaves and wavelengths in the visible, red edge and NIR regions of the spectrum. Any difference or variations in leaf structure, pigment concentrations or leaf water content will change the interaction between leaves and EMR.

Leaf anatomy is highly variable among plant species (Vogelmann 1989). There are significant structural differences in the cuticle and epidermal layer, palisade layer and spongy mesophyll tissue among plant species, causing them to absorb or reflect varying amounts of light in both visible and NIR regions of the spectrum (Gausman 1974). Species variation in spectral properties also can be attributed to differences in leaf pigmentation. For example, the absorption maximum of Chl a is at a wavelength that is 20 nm greater than that of Chl b (Guyot and Baret 1988). The proportion of Chl a to Chl b in leaves varies with genotypes. Therefore, if the relative proportion of Chl a were to increase, the maximum absorption corresponding wavelength for total Chl would shift to a greater wavelength, independent of total Chl concentration (Guyot and Baret 1988).

It is unlikely that an index or calibration equation developed for one species or study can be directly applied to another species or study (Richardson et al. 2002). From the perspective of both instrumentation and application, it is very important if we can identify a narrow waveband of "common" OW (OW_{Chl} and OW_N) and develop the "universal" indices for assessing Chl and N in leaves of a wide range of species.

2.3.4.2 Leaf water status

Reflectance of MIR region wavelengths is negatively correlated with water content of leaves. As water content decreases, MIR reflectance increases (absorption decreases). Because leaf pigments and structures transmit most MIR radiation, absorption is almost entirely related to the presence of water. Peak reflectance of wavelengths related to water absorption are at 1450 nm, 1780 nm, 1950 and 2500 nm (Curran 1989). Peñuelas et al. (1997) and Tian et al. (2001) pointed out that the strongest relationship

between water content and absorption in plant leaves occur between 1400 - 1900 nm. MIR reflectance is also a function of leaf thickness, as thicker leaves are capable of holding more water. A decrease in leaf water status caused by dehydration increases leaf reflectance in both visible and NIR regions of the spectrum (Carter 1994, Hoffer 1978, Hoffer and Johannsen 1969, Schepers 1996). Therefore, in order to improve the accuracy of Chl and N assessment, the effect of water variation should be taken into account. However, there are no reports on how to account for minimizing the influence of leaf water status on leaf Chl or N assessment.

2.3.4.3 Developmental stage

Plant leaf structure changes during leaf development. When comparing young leaves to mature leaves of healthy vegetation, young leaves have a lower reflectivity than the mature leaves. This is because young leaves are still developing structurally and are more compact with less air space in the mesophyll tissue than mature leaves (Gausman 1974). The air spaces within the mesophyll help to scatter (reflect and transmit) NIR radiation. Therefore, a mature leaf reflects more NIR radiation than a young leaf. Baret et al. (1987) noted that the general behavior of spectra from a wheat canopy over a growing season was independent of planting date and cultivar, but strongly dependent on the growth stage of the plants. Ahlrichs and Bauer (1982) found the strongest correlations between spectral data and plant parameters occurred during the initiation of tillering and anthesis. Understanding the effect of leaf or plant developmental stage on the interaction between leaves and spectral wavelengths can help increase the accuracy of Chl and N estimates by determining the optimal developmental stage for leaf measurement.

2.3.4.4 Stresses

The spectral characteristics of a leaf changes as it experiences stress. Increase in leaf reflectance of 400 – 2500 nm wavelengths is a consistent response to plant stress. Such increases tend to be spectrally similar among causes of stresses and species (Carter 1993). For example, Peñuelas et al. (1994) observed an increase in reflectance of 500 - 600 nm wavelengths in N-stressed sunflower leaves compared to unstressed leaves. Gamon et al. (1992) noted a similar pattern in reflectance of sunflower canopies, especially differences in reflectance between 8:00 a.m. and 12:00 p.m. between N stressed and unstressed plants.

The effect of stress on the interactions between leaves and light have been characterized for many stresses including insect or disease damage (Carter 1993, Carter 1994, Gausman 1974), light exposure (Schneckenburger and Schmidt 1996, Thiel et al. 1996), dehydration (Schepers et al. 1996, Carter 1993, Carter 1994), lack of nutrition (Schepers et al. 1996, McMurtrey III et al. 1996), frost damage (Sundblad et al. 2001), ozone damage (Carter 1993, Carter 1994), herbicide damage (Carter 1993, Carter 1994), insufficient mycorrhizae (Cantrell and Linderman 2001, Carter 1993, Carter 1994), senescence (Carter 1993, Carter 1994), etc. Any stress that causes a change in leaf optical properties will change the interaction between leaves and EMR by altering reflectance, transmittance or absorbance properties of leaves. Such changes are primarily caused by changes in plant leaf structure, such as cell wall degradation or wilting (Gausman 1974, Campbell 1996).

Stress can cause reduction in total Chl, which leads to changes in blue and red energy absorption and an increase in overall reflectance in the visible portion of the spectrum. Changes in red edge and NIR reflectance during periods of stress are often more noticeable than changes in the visible region of the spectrum. The concentration of Chl in leaves is a potential indicator of vegetation stress (Carter 1993, Carter 1994, Zarco-Tejada et al. 2000). Stress can alter the leaf structure and Chl concentration and thus affect the accuracy of Chl assessment. Spectral responses of leaves to light are similar among stresses and species (Carter 1993); therefore it may be possible to find some strategy to eliminate the stress effect on the accuracy of Chl assessment.

2.3.5 Optimum wavelength

2.3.5.1 The importance of optimum wavelengths

The importance of using OW_{Chl} for indices development is not recognized widely. Many indices have been developed for nondestructive assessment of Chl and N in several plant species (Curran et al. 1990, Gitelson & Merzlyak 1994a, Gitelson & Merzlyak 1996b, Gitelson et al. 1996a, Gitelson et al. 1996b, Blackburn 1998, Datt 1998, Datt 1999, Adams et al. 1999, Gamon & Surfus 1999). The accuracy of these indices, however, varies among plant species (Bullock and Anderson 1998, Peng et al. 1993, Schepers et al. 1992, Sunderman and Lamm 1991, Takebe and Yoneyama 1989) and developmental stage (Neilsen et al. 1995, Peng et al. 1993, Piekielek et al. 1995), and are affected by many factors including leaf thickness (Campbell et al. 1990, Chiariello et al. 1989, Neilsen et al. 1995, Osmond et al. 1989, Peng et al. 1993), concentrations of Chl and other pigments (Richardson et al. 2002), leaf water status (Martinez and Guiamet 2004) and other leaf

characteristics. One important reason for variation in the accuracy of these indices is the Chl-related wavelengths used in these indices are not the optimum wavelength (OW). To improve assessment accuracy of Chl or N, the OW selected for specific species should have a strong correlation with Chl or N concentrations (e.g. large R^2 and small RMSE) and be sensitive to changes in Chl or N across a wide range of concentrations and plant species.

2.3.5.2 Methods used for determining optimum wavelengths

The most common methods used for OW identification include: (1) regression (coefficient of determination (R^2) and root mean square error (RSME)), (2) first and second derivatives of reflectance values, and (3) reflectance difference and reflectance sensitivity.

2.3.5.2.1 Regression method

R^2 is an indicator and a measure of goodness-of-fit of linear regression and a summary measure of regression accuracy (Chatterjee et al. 2000). Theoretically, the OW selected for nondestructive Chl (OW_{Chl}) or N (OW_N) measurement should be based on regressions between spectral wavelength readings and either leaf Chl or N concentrations that have the largest R^2 and smallest RMSE. R^2 and RMSE have been used successfully for identification of OW_{Chl} using reflectance for some plant species (Carter and Spiering 2002, Gitelson et al. 1996a, Gitelson et al. 2003, Richardson et al. 2002). However, instead of analyzing the R^2 for the entire visible and NIR spectrum, most of the published results have focused only on analyzing R^2 of a few selected wavelengths (Moran et al. 2000, Schepers et al. 1996).

2.3.3.1.2 Derivative Methods

The use of derivatives is an effective analytical tool for characterizing or discriminating spectral bands that overlap other bands with different halfwidths. The 1st derivative of the spectra has been used widely in selection of Chl- or stress-related wavelengths and in indices development (Curran et al. 1990, Dixit and Ram 1985, Gitelson et al. 1996c, Gitelson et al. 2003, Moran et al. 2000, Morrey 1968, Richardson et al. 2002, Richardson et al. 2003). The 1st derivative of reflectance spectrum is also called first-difference spectrum and is calculated as $(R_n-R_{n-1})/(\lambda_n-\lambda_{n-1})$, here R = reflectance at wavelength n and λ=wavelength at n. The 1st derivative measures the amount of change in reflectance from one wavelength to the next in the raw reflectance spectrum (Richardson and Berlyn 2002). Derivatives have been successfully used for developing vegetation indices (VI) for remote sensing and for determining OW_{Chl} in the red edge of the spectrum (Dixit and Ram 1985). For example, the 1st derivative green VI and the 2nd derivative green VI can eliminate soil reflectance based on the linear nature of the relationship between canopy reflectance and spectral wavelength (Elvidge and Chen, 1995). Derivative indices were considered by Elvidge and Chen (1995) to be superior to ratio indices in determining plant ground cover over a variety of backgrounds. However, derivative transformation changes the original peak form by either creating non-meaningful peaks in the spectrum or eliminating some important peaks that might be related to Chl and/or N concentrations in leaves. The OW (OW_{Chl} and/or OW_{Chl}) selected by using 1st derivative is generally shifted from the real OW_{Chl} to either higher or lower wavelengths.

2.3.3.1.3 Reflectance difference and reflectance sensitivity methods

Reflectance difference is the difference of reflectance between the leaves from two treatments. Daughtry (2000) observed reflectance changes significantly near 550 nm, 715 nm, and at wavelengths longer than 750 nm, whereas reflectance differences in the blue (450 nm) and red (670 nm) were small (<1%) even though Chl concentration were three times greater. Reports for woody and herbaceous species indicate that reflectance differences in the NIR (>750 nm) were not related to leaf Chl concentration but were related to leaf structure (Knipling 1970, Chappelle et al. 1992, Gitelson and Merzlyak 1997).

The reflectance sensitivity at a given wavelength is computed by dividing the reflectance difference by the reflectance of a control (untreated sample) (Carter 1991, Carter 1993, Carter 1994, Carter et al. 1995). Reflectance difference and reflectance sensitivity has been used successfully in selection of OW for identification of various stresses (competition, herbicide, pathogen, ozone, insufficient mycorrhizae, dehydration) (Carter 1991, Carter 1993, Carter 1994, Carter et al. 1995, Daughtry 2000) and for evaluation of N concentrations in leaves (Moran et al. 2000). However, the reported reflectance sensitivity calculated by dividing the reflectance difference between the treated and the untreated samples by the reflectance of the untreated sample could not distinguish whether spectral difference is the result of difference in Chl concentration or caused by other factors. So it is important to verify the reflectance difference is caused by difference in Chl concentration rather than other factors when reflectance sensitivity is used for OW identification for Chl assessment.

A complete understanding the factors influencing the interactions between leaves and spectral wavelengths and how these factors affect the accuracy of nondestructive, leaf-level Chl and N assessment is necessary for (1) identifying reliable methods for selecting Chl- and N-related optimum wavelengths (OW_{Chl} and OW_N) for nondestructive Chl and N assessment; (2) analyzing the effect of methods and OW_{Chl} and OW_N used in indices development on accuracy of Chl and N assessment, (3) determining how the parameters of hand-held meters influencing the accuracy of Chl and N assessment; (4) developing hand-held meters with higher sensitivity for nondestructive Chl and N assessment than commercially available meters.

Table 2.1 Published indices used for leaf-level assessment of chlorophyll (Chl) and remote sensing for vegetation characterization

No	Indices	Index name	Detections[z]	References[y]
1	R_{750}/R_{700}	VI	Chl, stress	1, 2, 3, 4, 5, 6
2	R_{750}/R_{550}	VI	Chl	3, 6
3	R_{750}/R_{695}	VI	Chl	4
4	R_{740}/R_{720}	VI	Chl	4
5	$R_{NIR}/R_{705-715}$	VI	Chl	4
6	R_{750}/R_{556}			5
7	R_{850}/R_{710}	VI	Chl	7
8	R_{710}/R_{760}	VI	Stress	2
9	R_{695}/R_{760}	VI	Stress	2
10	R_{605}/R_{760}	VI	Stress	2
11	R_{695}/R_{420}	VI	Stress	2
12	R_{675}/R_{700}	VI	Chl	8
13	R_{800}/R_{680}	VI	Chl	9
14	R_{415}/R_{695}	VI	Chl	10
15	R_{415}/R_{710}	VI	N	10
16	R_{550}/R_{850}	VI	N and water stress	11
17	R_{650}/R_{850}	VI	N and water stress	11
18	R_{710}/R_{850}	VI	N and water stress	11
19	R_{550}/R_{950}	VI	N and water stress	11
20	R_{650}/R_{940}	VI	N and water stress	11
21	R_{710}/R_{940}	VI	N and water stress	11
22	Chl a: R_{800}/R_{675}	PSSR a	Chl a	11
23	Chl b: R_{800}/R_{650}	PSSR b	Chl b	11
24	$(R_{800}-R_{445})/(R_{800}-R_{680})$	SIPI	carotenoids	12, 13
25	$(R_{850}-R_{710})/(R_{850}-R_{680})$	NDVI	Chl, vegetation index	7
26	$(R_{800}-R_{700})/(R_{800}+R_{700})$	NDVI	Chl, vegetation index	14, 15
27	$(R_{800}-R_{680})/(R_{800}+R_{680})$	NDVI	Chl, vegetation index	11
28	$(R_{750}-R_{675})/(R_{750}+R_{675})$	NDVI	Chl, vegetation index	16, 17
29	$(R_{531} - R_{570})/(R_{531} + R_{570})$	PRI	xanthophyll	13, 18, 19
30	$(R_{750}-R_{680})/(R_{750}+R_{690})$	NDVI	Chl, vegetation index	21
31	$(R_{750}-R_{705})/(R_{750}+R_{705})$	Chl NDI	Chl	3, 4, 14, 21
32	$(R_{750}-R_{800})/(R_{695}-R_{740})-1$	NDVI	Chl, vegetation index	22
33	$\int_{705}^{750}(R_{\lambda}/R_{705}-1)d\lambda$	RII	Chl	14, 20
34	$\sum_{\lambda_1}^{\lambda_n}\rho(\lambda_i)/\Delta\lambda_j$	FDGVI	vegetation index	23

[z]Stress: competition, herbicide, pathogen, ozone, mycorrhizae, island, senescence, dehydration

[y]Carter, 1993 (1), Carter, 1994 (2); Gitelson et al. 1996a (3), 1996b (4), 1996 (5); Lichtenthaler et al. 1996 (6); Datt . 1999 (7); Chappelle et al 1992 (8); Blackburn 1998 (9); Read et al 2002 (10); Schepers et al 1996 (11); Moran et al. 2000 (12); Peñuelas et al. 1995b (13); Gitelson and Merzlyak 1994a (14), 1994b (15); Gamon and Qiu 1999 (16); Richardson and Berlyn 2002 (17); Gamon et al. 1992 (18), 1997 (19); Richardson et al.2002 (20); Gamon and Surfus 1999 (21); Gitelson et al 2003 (22); Elvidge and Chen 1995 (23)

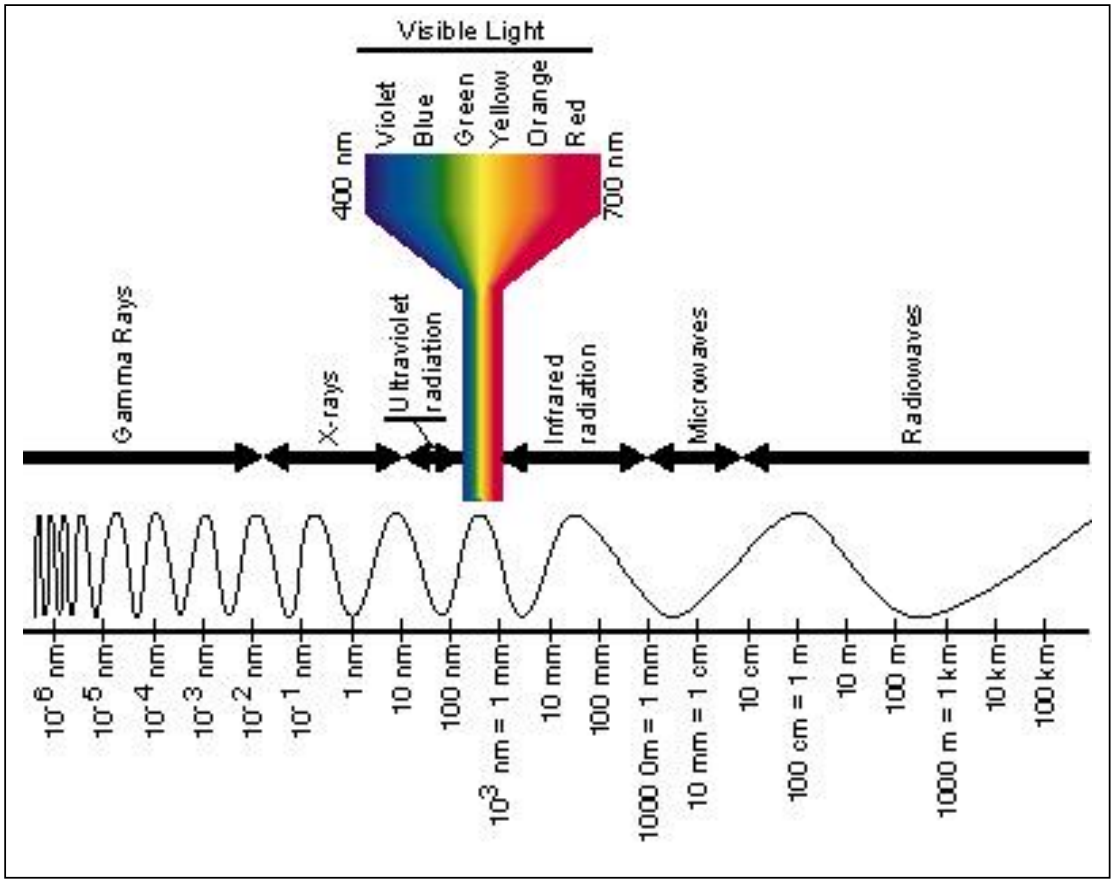

Figure 2.1. Lights and their properties as electromagnetic radiation (EMR) (Kaufmann 1991)

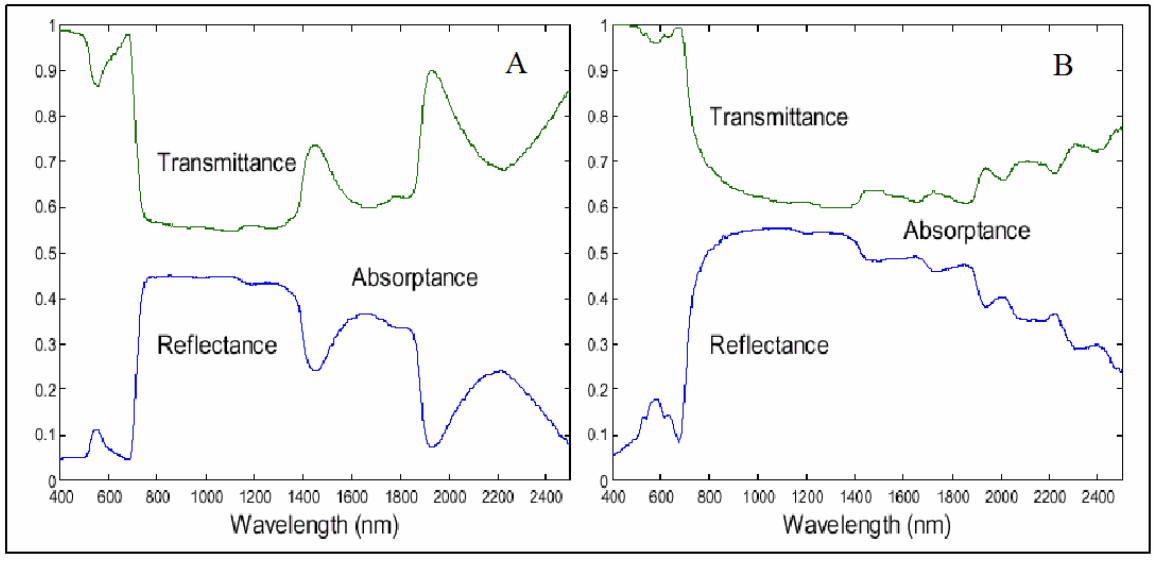

Figure 2.2. Reflectance and transmittance spectra of (A) fresh and (B) dry poplar leaves (Jacquemoud and Ustin 2001)

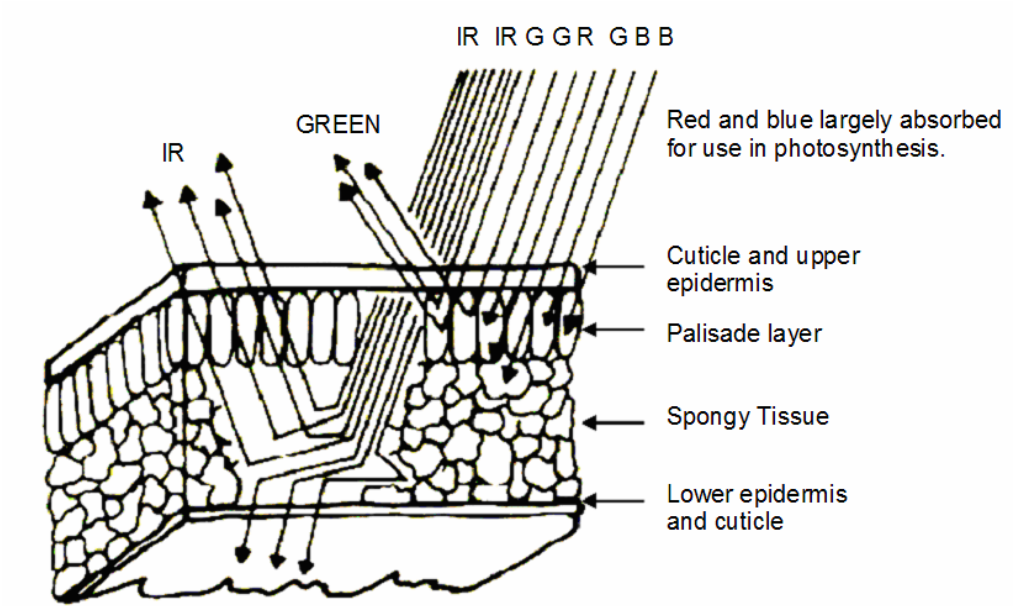

Figure 2.3. Red (R), green (G), blue (B), and infrared (IR) electromagnetic radiation (EMR) interacting with structural components of a leaf (Campbell 1996)

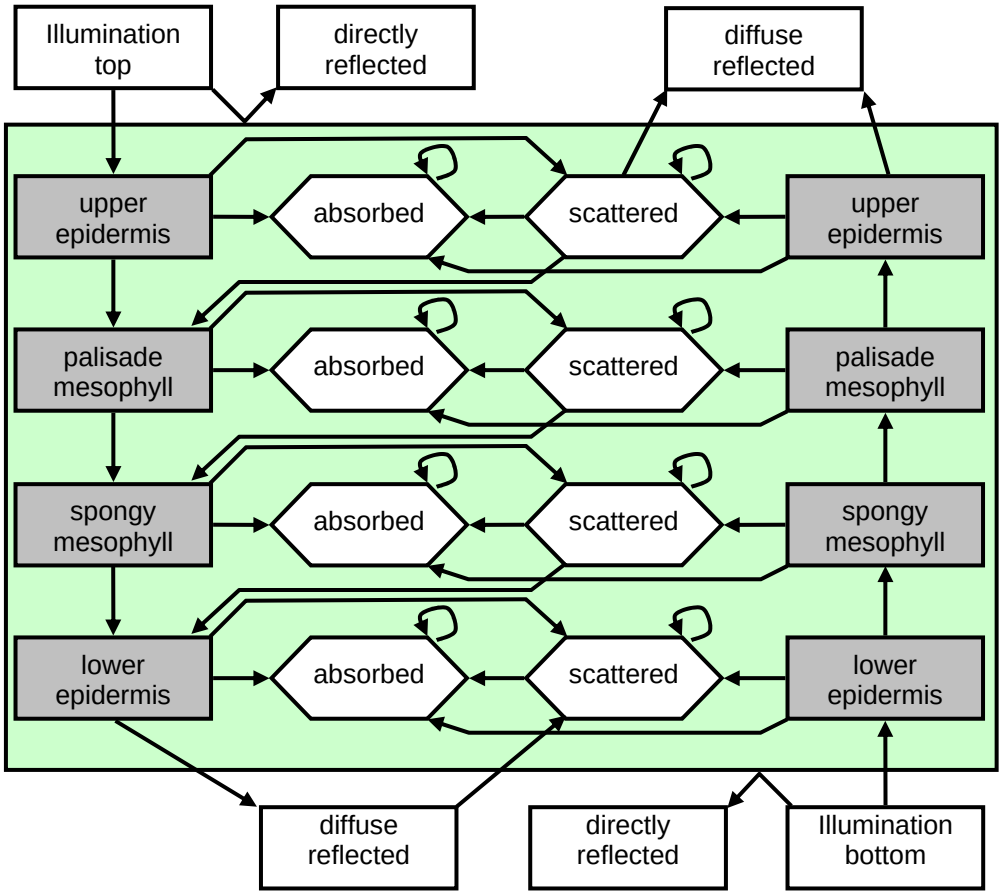

Figure 2.4. Stochastic radioactive transfer model of interactions between leaf structural components and electromagnetic radiation (EMR) (Jacquemoud and Ustin 2001)

CHAPTER 3

SIMPLE LINEAR REGRESSION AND REFLECTANCE SENSITIVITY ANALYSIS USED TO DETERMINE THE OPTIMUM WAVELENGTH FOR THE NONDESTRUCTIVE ASSESSMENT OF CHLOROPHYLL IN FRESH LEAVES USING SPECTRAL REFLECTANCE

3.1 Abstract

The accuracy of nondestructive optical methods for chlorophyll (Chl) assessment based on leaf spectral characteristics depends on the wavelengths used for Chl assessment. To determine the optimum wavelengths for Chl assessment (OW_{Chl}) using reflectance spectroscopy, almond (*Prunus dulcis* (Mill.) D.A. Webb 'Nonpareil'), poplar (UCC-1, a hybrid of *Populus trichocarpa* × *P. deltoides*, Union Camp, Princeton, NJ) and apple (*Malus domestica* Borkh 'Fuji') trees were grown at different rates of nitrogen (N) fertilization to produce leaves with different Chl concentrations. Spectral reflectance of leaf discs was measured using a spectroradiometer (300 nm to 1100 nm at 1 nm intervals), and total Chl concentration in leaf discs was determined. The OW_{Chl} for nondestructive Chl assessment by reflectance spectroscopy was identified using three methods (1) the coefficient of determination (R^2) from simple linear regression, (2) reflectance sensitivity analysis, and (3) the 1st spectral derivative method. Our results indicated that the 1st derivative method can be used to estimate OW_{Chl} in the red edge region and reflectance sensitivity analysis can be used to estimate the OW_{Chl} in both the red edge and visible regions. Reflectance sensitivity analysis was more accurate when used for OW_{Chl} selection than the 1st derivative method because methods used for reflectance sensitivity analysis ensures that differences in the spectral reflectance are caused by differences in Chl concentration, while the 1st derivative method could not distinguish whether spectral

differences were the result of differences in Chl concentration or caused by other factors. However, neither the 1st derivative method nor reflectance sensitivity analysis alone could accurately identify the actual OW_{Chl}. R^2 was a useful indicator for verifying the accuracy of OW_{Chl} selection. Higher R^2 values were usually associated with lower root mean square errors (RMSE) and higher reflectance sensitivity; therefore the wavelengths with the highest R^2 and highest reflectance sensitivity were selected as the OW_{Chl}. Our results indicate that using simple linear R^2 in combination with reflectance sensitivity analysis is the more reliable method for determining OW_{Chl} in plant leaves.

3.2 Introduction

The chlorophylls, chlorophyll a (Chl a) and chlorophyll b (Chl b), are essential pigments for the conversion of light energy to stored chemical energy in plants and their presence and function is important from both physiological and applied perspectives (Buschmann et al. 1994, Carter 1998, Gitelson et al. 2003, Pinar and Curran 1996, Richardson et al. 2002). The amount of solar radiation absorbed by a leaf is largely a function of the foliar concentration of photosynthetic pigments, and thus Chl concentration can directly limit photosynthetic potential and primary production (Curran et al. 1990, Filella et al. 1995). Most of leaf N is incorporated in Chl; therefore Chl concentration gives an indirect estimation of plant N status (Filella et al. 1995, Moran et al. 2000). Leaf Chl concentration is also closely related to plant stress and can be used as an indicator of plant stress (Carter and Knapp 2001, Hendry 1987, Peñuelas and Filella 1998).

Traditionally, leaf Chl extraction with organic solvents and spectrophotometric determination of the extract was the standard method used for Chl analysis (Arnon1949). Recently, alternative nondestructive optical methods for Chl assessment, based on the absorbance and/or reflectance of light by the intact leaf, have been developed (Curran et al. 1990, Adams et al. 1999, Datt 1999, Gamon and Surfus 1999, Markwell et al. 1995). These optical methods require no chemical analysis, are nondestructive, simple to use, fast, inexpensive and can be used in the field (Buschmann and Nagel 1993, Gitelson and Merzlyak 1994, Gitelson et al. 1996a, Gitelson et al.1996b, Markwell et al. 1995). The most common optical methods for estimating leaf Chl concentrations are based on the use of either (1) specific Chl-related wavelengths (i.e. 550, 698, 692, or 695 nm) (Thomas and Gausman 1977, Jacquemoud and Baret 1990, Carter 1994, Carter 1998, Moran and Moran 1998) or (2) a Chl-related wavelength in combination with a Chl-insensitive wavelength in the form of a wavelength ratio (i.e. R_{698}/R_{760}) or specific indices or algorithms [e.g. $(R_{800}-R_{445})/(R_{800}-R_{680})$] (Moran et al. 2000, Peñuelas et al. 1995).

Previous work has mainly focused on developing and evaluating Chl-related indices for nondestructive optical assessment of Chl (Curran et al. 1990, Gitelson and Merzlyak 1994, Gitelson and Merzlyak 1996, Gitelson et al. 1996a, Gitelson et al. 1996b, Blackburn 1998, Datt 1998, Datt 1999; Adams et al. 1999, Gamon and Surfus 1999); however, the applicability of the proposed indices were seldom tested using a second, independent, data set. Most published indices rarely have been tested using data from species other than those used in the formulation of the index (Richardson et al. 2002). In some cases, published indices are not presented in a manner that allows meaningful comparison between different studies (Gitelson et al. 2003, Richardson et al. 2002).

There are many reasons why reported indices or algorithms are not applicable for Chl assessment across different studies. However, one of the main reasons is that the optimum wavelengths for measuring Chl (OW_{Chl}) used in one study differed from the OW_{Chl} used in other studies. Differences in OW_{Chl} between studies are a result of variation in leaf properties among plant genotypes and phenotypes and optical characteristics of plant leaves. In many studies, the most common technique used to select the OW_{Chl} for developing Chl-related indices is the use of the first or second derivative of the Chl spectra (Curran et al. 1990, Dixit and Ram 1985, Gitelson et al. 1996, Gitelson et al. 2003, Morrey 1968, Richardson et al. 2002). These derivatives are useful analytical tools for characterizing or discriminating one analytic band that is overlapped by other bands with different halfwidths. Derivatives can be used to resolve or enhance smaller peaks that are incompletely resolved from larger peaks due to either the background or noise (Dixit and Ram 1985, Moran et al. 2000, Morrey 1968, Curran et al. 1990). However, derivatives change the original peak form and may eliminate some important peaks.

Substances or molecules (e.g. Chl) have specific absorption or reflection spectra. Based on spectral changes in absorption, transmission or reflectance, Chl can be identified and quantified at specific wavelengths. Specific wavelengths have different levels of spectral (reflectance and/or transmission) sensitivity and accuracy for measuring Chl. Reflectance sensitivity explains how sensitive the reflectance is at a specific wavelength for measuring Chl, whereas R^2 is a measure of 'goodness-of-fit' of the linear regression of wavelength response and Chl concentration. Theoretically, the OW_{Chl} for a nondestructive Chl measurement should have the highest reflectance sensitivity and

largest R^2. Although R^2 has been widely used in laboratory quantitative analysis, only few studies have been reported using R^2 from reflectance spectra for Chl-related waveband identification (Gitelson et al. 2003). Reflectance sensitivity has been used to identify stress-sensitive Chl-related wavelengths (Carter 1993, Carter 1994, Moran et al. 2000) but not used in combination with R^2 to determine OW_{Chl}. Our objectives were to (1) evaluate whether simple linear regression in combination with reflectance sensitivity analysis can be used to determine OW_{Chl} for Chl assessment using reflectance, and (2) compare the accuracy of estimates from a combined regression and reflectance sensitivity analysis to those obtained from the widely used 1^{st} spectral derivative method.

3.3 Materials and methods

3.3.1. Plant materials

In 1999, 2000, and 2002, trees of almond (*Prunus dulcis* (Mill.) D.A. Webb 'Nonpareil'), poplar (UCC-1, a hybrid of *Populus trichocarpa* × *P. deltoides*, Union Camp, Princeton, NJ) and apple (*Malus domestica* Borkh 'Fuji') on M.26 rootstocks were grown in 7.2L pots containing 1 peat moss:2 pumice:1 sandy loam soil (v:v) in a lathhouse in Corvallis, Oregon, (44° 30' N, 123° 17' W) from March to June. Beginning from budbreak in early May, trees were fertilized every 2 weeks with 10.7 mM N, using Plantex® 20N-10P_2O_5-20K_2O water-soluble fertilizer with micronutrients (Plantex Corp.,Ontario, Canada). When new shoots were approximately 15 cm long, plants were moved to full sunlight and fertilized weekly with Plantex® for 3 weeks. Beginning in July, plants were fertilized twice weekly with one of six N concentrations (0, 2.5, 5, 7.5,

10, or 20 mM N from NH_4NO_3) by applying 300 ml of a modified Hoagland's solution (Hoagland and Arnon, 1950) to each pot until the end of September.

3.3.2 Spectral reflectance and Chl determination

In August and September, 12 fresh leaves from each species (genotype) in each N fertigation treatment were removed from trees, discs were excised from leaves with a cork borer (2.85 cm^2), and spectral reflectance of leaf discs was determined from 300 nm to 1100 nm at 1 nm intervals using a Li-1800 spectroradiometer (Li-Cor Inc., Lincoln, NE). After scanning, each leaf disc was cut into smaller pieces, placed in a test tube, and extracted in 80% (v/v) acetone at 4 °C in the dark for 24 h. Absorbance of the extract was measured with a UV-visible spectrophotometer (Shimadzu UV-1601, Shimadzu Scientific Instruments, Inc., Columbia, MD) and total Chl concentration was calculated according to Lichtenthaler and Wellburn (1983). Total Chl concentration of leaves in the different N fertigation treatments ranged from 261 to 1188µmol.m^{-2} in 'Fuji' apple, 160 to 659µmol.m^{-2} in poplar, and 173 to 710µmol.m^{-2} in almond.

3.3.3 Regression analyses of spectral reflectance and Chl data

Using Microsoft Visual Basic 6.0 (Microsoft Corp., Redmond, WA), a customized software application was developed to directly perform simple linear regression (linear-least-squares-fit) and calculate root mean square error (RMSE) and coefficients of determination (R^2) between the spectral reflectance reading at 1 nm intervals from 300 nm to 1100 nm and Chl concentrations (Chl a, Chl b or Chl a+b) in leaf discs. The R^2 of the reflectance vs. Chl relationship for Chl a, Chl b and Chl a+b at

each wavelength was used to generate R^2-curves (wavelength of reflectance measurement vs. R^2 at each wavelength) for predicting the actual OW_{Chl} for estimating Chl concentrations using reflectance. The RMSE of the reflectance vs Chl relationship for Chl a, Chl b and Chl a+b at each wavelength was used to generate RSME-curves (wavelength of reflectance measurement vs. RSME at each wavelength) to validate the strength of using R^2-curves for predicting the actual OW_{Chl} for estimating Chl concentrations.

3.3.4 Reflectance sensitivity analysis

Reflectance sensitivity measures changes of leaf spectral reflectance based on unit change in leaf Chl concentration. Reflectance sensitivity analysis was performed using four single leaf samples per genotype selected from N fertigation treatment (S1, S2, S3 and S4) with different total Chl concentrations. Reflectance curves for wavelengths at 1 nm increments from 300 nm to 1100 nm were developed for (1) 'Fuji' apple leaves with Chl concentrations of 355 (S1), 439 (S2), 676 (S3), and 1093 (S4) $\mu mol.m^{-2}$; (2) poplar leaves with Chl concentrations of 185 (S1), 234 (S2), 326 (S3), and 659 (S4) $\mu mol.m^{-2}$; and almond leaves with Chl concentrations of 173 (S1), 249 (S2), 367 (S3), or 660 (S4) $\mu mol.m^{-2}$.

Within a genotype, the reflectance values for leaf samples S2, S3 and S4 were subtracted from the reflectance for leaf sample S1 (i.e., R1-R2; R1-R3; and R1-R4) to generate reflectance difference values for each measured wavelength. Reflectance difference curves were developed based on wavelength vs. reflectance difference values at 1 nm intervals from 300 nm to 1100 nm. Sensitivity curves based on differences in

reflectance were generated by dividing the reflectance difference value from reflectance difference curves by the reflectance of the leaf sample with the lowest Chl concentration [i.e., (R1-R2)/R1; (R1-R3)/R1; and (R1-R4)/R1]. These resultant sensitivity values were then plotted against the wavelength at which each reflectance measurement was taken (e.g. wavelength vs. sensitivity). Sensitivity curves based on 100 $\mu mol \cdot m^{-2}$ differences in Chl a were generated by dividing the reflectance difference value from the reflectance difference curves by the difference in Chl a concentration between the leaf sample with lowest Chl concentration (Chla1) and the leaf samples with higher Chl concentrations {i.e., [(R1-R2)/(Chla2-Chla1)]*100; [(R1-R3)/(Chla3-Chla1)]*100; and [(R1-R4)/(Chla4-Chla1)]*100}. The resultant sensitivity values based on 100 $\mu m \cdot m^{-2}$ differences in Chl a were then plotted against the wavelength at which each reflectance measurement was taken (e.g. wavelength vs. sensitivity). Sensitivity curves for differences in Chl b and Chl a+b were generated in a similar manner as described for Chl a.

3.3.5. 1st derivative method

The 1st-derivative spectral curve measures the change in reflectance from one wavelength to the next; it is a measure of the slope of the raw values of the reflectance spectrum (Richardson and Berlyn 2002). The 1st derivation of reflectance spectrum is calculated as $(R_n-R_{n-1})/(\lambda_n-\lambda_{n-1})$, here R_n is the reflectance value at wavelength n, and λ_n is the wavelength. The 1st derivative of the data from the reflectance spectra was used to generate a 1st-derivate curve (wavelength vs. 1st derivative of reflectance at each wavelength).

3.4 Results and discussion

3.4.1 Regression analyses of spectral reflectance and Chl data

The coefficient of determination (R^2) is a measure of 'goodness-of-fit' of linear regression and a summary measure of regression accuracy (Chatterjee et al. 2000). The R^2-curves indicated that the relationship between reflectance values and leaf Chl concentrations at different wavelengths can be used to predict the actual OW_{Chl} for Chl assessment (Figure 3.1). Maximum (peak) R^2 values fell in three regions: UV (380 - 440 nm), visible (520 - 600 nm), and red edge (690 – 740 nm) (Table 3.1). The peak R^2 values in the red edge and visible regions for Chl a, Chl b, and Chl a+b were much larger than the peak R^2 values in the UV region for all genotypes, indicating that OW_{Chl} selected from these two regions has a higher accuracy over the OW_{Chl} selected from the UV region. A few limited studies also showed that R^2 is a useful tool used for Chl-related waveband identification (Gitelson et al. 2003, Read et al. 2002). Larger R^2 values were usually associated with smaller RMSE. The peaks on RMSE-curves were inverted compared to the peaks on the R^2-curves (Figure 3.1). Peaks with the largest R^2 values had the smallest RMSE, validating that R^2 was a reliable parameter for selecting OW_{Chl} for Chl assessment. Our results also showed that simple linear regression is better than multiple and polynomial regressions when used for OW_{Chl} identification, because both multiple and polynomial regressions either generated some non-meaningful R^2 peaks or eliminated some important peaks R^2 as well as shifted the OW_{Chl} either to higher or lower wavelengths than actual OW_{Chl} (results not shown).

The simple linear regression largest R^2 and the corresponding OW_{Chl} (for Chl a, Chl b or Chl a+b) varied among genotypes (Table 3.1). Moreover, the OW_{Chl} for

measuring different Chl (Chl a, Chl b and Chl a+b) within the leaves of the same genotype were also different. The peak R^2 and the corresponding wavelengths of Chl a+b were between the peak R^2 and the corresponding wavelengths of Chl a and Chl b, but tended to be closer to the peak R^2 and the corresponding wavelength of Chl a, respectively (Table 3.1). These results indicate that the simple linear regression method can accurately identify the proper OW_{Chl} for assessing concentrations of specific Chl types (Chl a, Chl b and Chl a+b) in various plant genotypes. The R^2 peak related wavelengths for different genotypes fall in three narrow regions: UV (380 - 440 nm), visible (520 - 600 nm), and red edge (690 – 740 nm) (Figure 3.1A-C, Table 3.1). A "common" OW_{Chl} from an overlapping region among genotypes from either visible (550 -580 nm) or red edge (700 – 730 nm) can be used to assess Chl across species, although it is not as accurate as using the OW_{Chl} derived for specific genotypes.

3.4.2. Reflectance sensitivity analysis

The original reflectance spectra for 'Fuji' apple (Figure 3.2A), poplar (Figure 3.3A), and almond (Figure 3.4A) leaves showed only one reflectance peak in the visible wavelength region of 550 - 560 nm. Reflectance difference curves derived from the original reflectance spectra showed two peaks: one in the red edge region (710 – 730 nm) and the other in the visible region (560 - 600 nm) (Figure 3.2B, Figure 3.3B, Figure 3.4B). Peaks in the red edge region were larger but much narrower than peaks in the visible region. Similar results have been reported when using spectral reflectance to identify the interactions between leaves and spectral reflectance caused by stresses and N fertilization (Carter 1994, Moran et al. 2000). A larger and narrower peak of reflectance

difference in the red edge region enables selection of OW_{Chl} from the red edge region to develop sensitive, accurate and reliable indices for Chl assessment.

Reflectance sensitivity analysis showed similar trends in curves of reflectance sensitivity based on referential reflectance (Figure 3.2C, Figure 3.3C, and Figure 3.4C) or 100μmol.m^{-2} differences in Chl a, Chl b or Chl a+b (Figure 3.2D-F, Figure 3.3D-F, and Figure 3.4D-F). Two peaks in the reflectance sensitivity curves were similar to peaks in the reflectance difference curves: one was in red edge region (706 - 716 nm) and the other was in visible region (560 - 600 nm). Reflectance sensitivity based on referential reflectance has been used to identify stress-sensitive wavelength (Carter 1993, Carter 1994) and the effect of N fertilization (Moran et al. 2000). Our results showed that reflectance sensitivity based on differential reflectance or 100μmol.m^{-2} differences in Chl a, Chl b or Chl a+b can be used for selecting the OW_{Chl} for Chl assessment.

When the difference in reflectance between samples was caused by a difference in leaf Chl concentration, reflectance sensitivity based on referential reflectance was similar to that based on differences in leaf Chl concentration; when the difference in reflectance between samples was caused by other factors instead of a difference in Chl concentrations, reflectance sensitivity based on referential reflectance was different from that based on differences in leaf Chl concentration and could not used for OW_{Chl} selection (Figure 5.1, Figure 5.2). Our results confirmed the hypothesis that reflectance sensitivity based on 100μmol.m^{-2} differences in Chl a, Chl b or Chl a+b is more accurate than reflectance differences and reflectance sensitivity based on referential reflectance; because reflectance sensitivity based on differences in Chl concentration ensures that the difference in spectral reflectance is caused by differences in Chl concentration, while

differences in spectral reflectance alone could not tell whether the differences are caused by differences in Chl concentration or by other factors.

Peak wavelengths based on reflectance difference curves (Figure 3.2B, Figure 3.3B, and Figure 3.4B) and wavelength sensitivity curves (Figure 3.2C-F, Figure 3.3C-F, and Figure 3.4C-F) differed among the genotypes we analyzed. Within a genotype, the peak wavelengths in the visible and red edge regions for either reflectance difference or wavelength sensitivity curves were similar (Figure 3.2D-F, Figure 3.3D-F, Figure 3.4D-F). This demonstrates that both reflectance difference and wavelength sensitivity methods can be used to select OW_{Chl} for assessing Chl in leaves with a wide range of Chl concentrations.

3.4.3. 1st derivative method

The 1st derivative is very useful for characterizing or discriminating one spectral band that is overlapped by other bands with different halfwidths (Dixit and Ram 1985). However, we found that the 1st derivative transformation of reflectance spectra from leaves of three plant genotypes changed the original peak form by either generating some non-meaningful peaks or eliminating some important peaks that might be Chl-related (Figure 3.5D-F). After the 1st derivative transformation, the transformed reflectance spectra contained five peaks (Figure 3.5D-F). Only one of these five peaks on the 1st derivative curves, in the red edge region, was sensitive to Chl concentrations in leaves and this peak was in a similar region as a peak found in the R^2 and reflectance sensitivity curves. The other four peaks occurred at wavelengths that were not related to any peaks in either the reflectance sensitivity curves or the R^2-curves and were not sensitive to Chl

concentrations in leaves. Furthermore, the OW_{Chl} selected for Chl assessment using the 1st derivative method was shifted either to a higher or lower wavelength than the actual OW_{Chl} selected using R^2-curves. The 1st derivative of reflectance spectra from leaves has been used widely to assess plant stress and to identify Chl-related wavelength in the red edge for Chl-related indices development (Curran et al. 1990, Dixit and Ram 1985, Gitelson et al. 1996, 2003, Morrey 1968, Richardson et al. 2002), but no reports have described how peak shifts caused by 1st derivative transformation influence the accuracy of OW_{Chl} identification and Chl assessment. We found that using the 1st derivative method alone was not the most accurate method to determine the OW_{Chl} for Chl assessment. For example, the variation of OW_{Chl} selected in the region of red edge using 1st derivative method was 26 nm (703 - 726nm) in 'Fuji' apple leaves with Chl concentration 355-1093µmol.m^{-2}, 20 nm (695 - 715 nm) in poplar leaves with Chl concentration 185-659 µmol.m^{-2} and 16 nm (697 - 713 nm) in almond leaves with Chl concentration173-660 µmol.m^{-2} (Figure 3.5D-F). This variation is big enough to impair the ability of 1st derivative method to accurately identify OW_{Chl}.

3.4.4. Comparisons of R^2, reflectance sensitivity and 1st derivative methods

Comparison of reflectance sensitivity (Figure 3.6A), R^2 (Figure 3-6B) and the 1st-derivative (Figure 3.6C) curves, for 'Fuji' apple, poplar and almond showed there were two peaks related to leaf Chl concentrations in reflectance sensitivity and R^2 curves but only one in the 1st-derivative curve. One Chl-sensitive peak in the reflectance sensitivity and R^2 curves was in the visible region, and the other in the red edge region. The only Chl-sensitive peak in the 1st-derivative curve was in the red edge region. In general, the

peak in the red edge region of the R^2-curves or reflectance sensitivity curves was shaper and narrower than the peak in the visible range. Peak wavelengths in the red edge region obtained by the different methods (R^2, reflectance sensitivity or 1^{st}-derivative) were between 690 nm -750 nm.

The corresponding OW_{Chl} for the apexes of the curves obtained by different methods for measuring Chl concentrations within the same genotype were different. The OW_{Chl} in the red edge region identified by R^2-curves for 'Fuji' apple, poplar and almond were 717 nm, 720 nm, and 710 nm, respectively, and the OW_{Chl} identified by reflectance sensitivity curves for the same samples were 717 nm, 708 nm, and 705 nm, respectively. The 1^{st}-derivative curves identified OW_{Chl} for the same samples at 726 nm, 713 nm, and 702 nm, respectively. In the visible region the OW_{Chl} identified by R^2-curves for measuring Chl concentrations in 'Fuji' apple, poplar and almond were 552 nm, 574 nm, and 549 nm, respectively, while the OW_{Chl} identified by reflectance sensitivity analysis for the same samples were 558 nm, 599 nm and 564 nm, respectively (Figure 3.6A-B).

Optimum wavelengths obtained by using reflectance sensitivity or 1^{st}-derivative curves were either at higher or lower wavelengths than the OW_{Chl} obtained using R^2-curves. There was no consistent trend that could be used for predicting which method would obtain higher or lower values. The high or low OW_{Chl} specified by reflectance sensitivity or 1^{st}-derivative curves could cause some error in determining the OW_{Chl} for Chl assessment. For example, the OW_{Chl} selected for measuring total Chl (Chl a+b) in leaves of poplar by the methods of R^2, reflectance sensitivity and 1^{st} derivative were 720 nm, 708 nm, and 713 nm, respectively. The R^2 of simple linear regressions at the OW_{Chl} selected by using R^2-curve is (720 nm) slightly larger than the OW_{Chl} selected by using

reflectance sensitivity (708 nm) and 1st derivative curves (713 nm), respectively, although there is no significant statistic difference among the R^2 selected by different methods (Figure 3.7A-C).

The OW_{Chl} selected using either the reflectance sensitivity analysis or 1st-derivative was shifted either to higher or lower wavelengths than the actual OW_{Chl} selected using R^2-curves (Figure 3.6A-B); however the OW_{Chl} selected by using reflectance sensitivity method was more accurate and meaningful than selected by using the 1st derivative method. The accuracy of reflectance sensitivity analysis based on differences in Chl concentration was greater than the 1st derivative method because the methods used for reflectance sensitivity analysis ensure the differences in the spectral reflectance are caused by differences in Chl concentration, while the 1st derivative method could not distinguish whether spectral differences were caused by differences in Chl concentration or by other factors. Furthermore, we found that the OW_{Chl} for Chl assessment determined by reflectance sensitivity analysis does not vary within the same plant genotype while the OW_{Chl} selected using the 1st derivative method varied both within and between genotypes (Figure 3.1D-F, Figure 3.3D-F, Figure 3.4D-F and Figure 3.5D-F). All these results provide support that reflectance sensitivity analysis is better than the 1st derivative data transformation used for OW_{Chl} identification.

3.5. Conclusions

Theoretically, the OW_{Chl} selected for nondestructive Chl measurement should be based on having the highest reflectance sensitivity and largest R^2 for the regression between spectral wavelength readings and leaf Chl concentrations. We found that larger

R^2 values were usually associated with smaller RMSE (Figure 3.1) and higher reflectance sensitivity in measuring leaf Chl. When selecting OW_{Chl} for Chl assessment our results indicate that it is best to use the R^2 from simple linear regression in combination with reflectance sensitivity for determining the OW_{Chl}. The combination of R^2 and reflectance sensitivity was a reliable method for determination of the OW_{Chl} for Chl measurement as well as for other pigments and N assessment in both transmission (see results in Figure 6-2 to Figure 6-5) and reflectance spectroscopy.

3.6 References

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Table 3.1 Maximum (peak) coefficients of determination (R^2) values and corresponding wavelengths (λ) for the relationship between chlorophyll concentration in leaves and reflectance values at 1 nm intervals from 300 to 1100 nm.

		UV region		Visible region		Red-edge region	
Genotype[z]	Chlorophyll[y]	λ (nm)	R^{2x}	λ (nm)	R^2	λ (nm)	R^2
Apple	Chl a	412	0.5264 aABC *	552	0.7785 bAB **	720	0.9198 cBCD ***
	Chl b	390	0.4759 aAB *	550	0.7225 bA **	717	0.8465 bABC ***
	Chl a+b	410	0.5236 aABC *	552	0.7695 bAB **	720	0.9072 cBC ***
Poplar	Chl a	422	0.7474 aC **	581	0.9497 bD ***	715	0.9579 bD ***
	Chl b	423	0.6134 aB **	563	0.7440 bAB **	730	0.7801 bA **
	Chl a+b	422	0.7331 aC **	574	0.9166 bCD ***	720	0.9352 bCD ***
Almond	Chl a	420	0.2632 aA ^{ns}	549	0.8740 bBC ***	710	0.8678 bABC ***
	Chl b	420	0.3121 aA *	558	0.8256 bABC ***	710	0.8144 bAB ***
	Chl a+b	420	0.2757 aA ^{ns}	549	0.8737 bBC $^{* **}$	710	0.8667 bABC ***

[z] Apple = *Malus domestica* 'Fuji'; Poplar = *Populus trichocarpa* x *P. deltoids*; Almond = *Prunus dulcis* 'Nonpareil'.

[y] Chl a = chlorophyll a; Chl b = chlorophyll b; Chl a + b = total chlorophyll

[x] ns – not significant ($p>0.05$), * $p<0.05$, ** $p<0.01$, *** $p<0.001$; R^2 followed by the same lower case letter within a row or upper case letter within a column are not significantly different ($p<0.05$, Fisher's Z-Test, n=72)