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

**PINGHAI DING**

AN ABSTRACT OF THE DISSERTATION OF

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Abstract approved:

Leslie H. Fuchigami

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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APPROVED:

Major Professor, representing Horticulture

Head of the Department of Horticulture

Dean of the Graduate School

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Pinghai Ding, Author

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

## 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 $\lambda_n$ 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).

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

### *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).

2.3.5.2.2 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.

2.3.5.2.3 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)

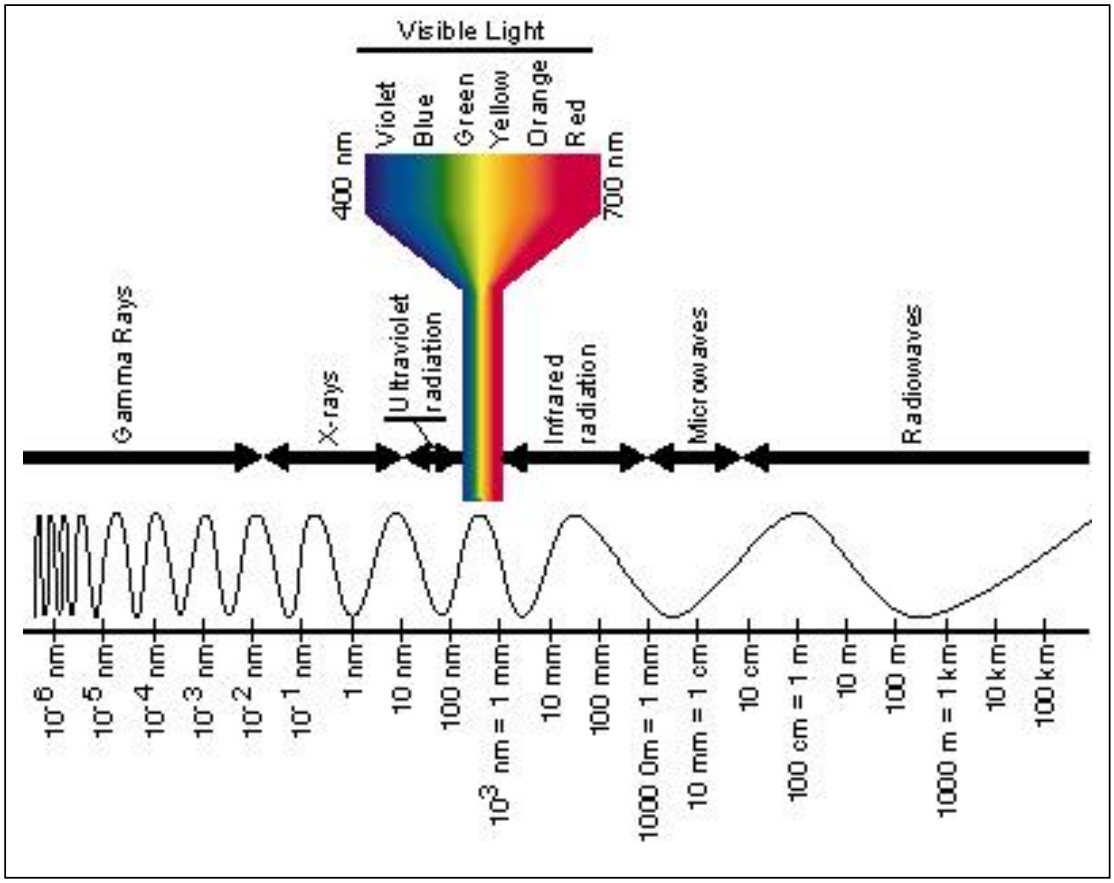


Figure 2.1. Lights and their properties as electromagnetic radiation (EMR) (Kaufmann 1991)

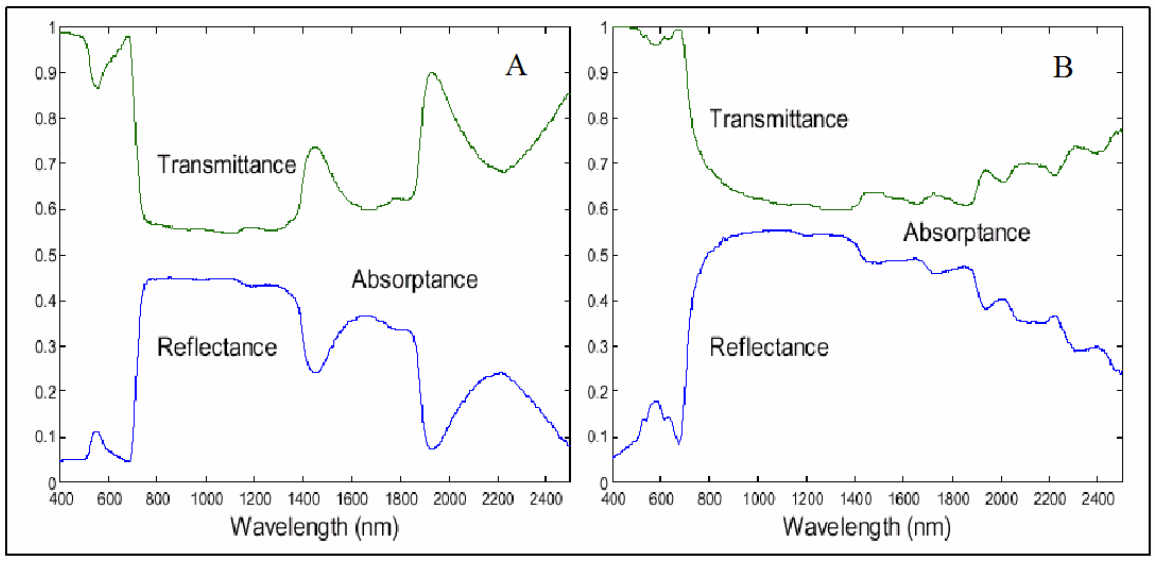


Figure 2.2. Reflectance and transmittance spectra of (A) fresh and (B) dry poplar leaves (Jacquemoud and Ustin 2001)

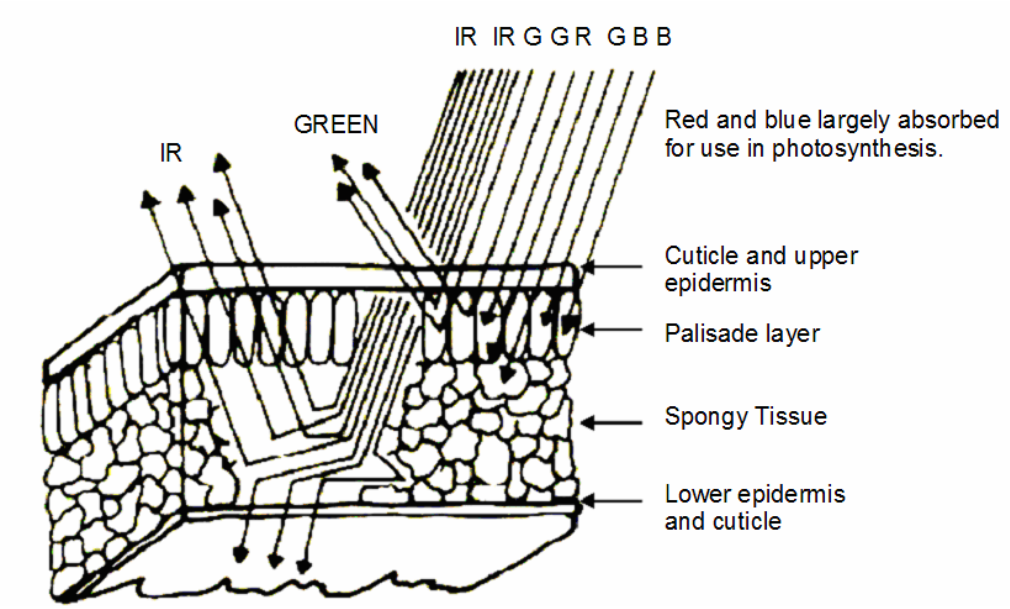


Figure 2.3. Red (R), green (G), blue (B), and infrared (IR) electromagnetic radiation (EMR) interacting with structural components of a leaf (Campbell 1996)

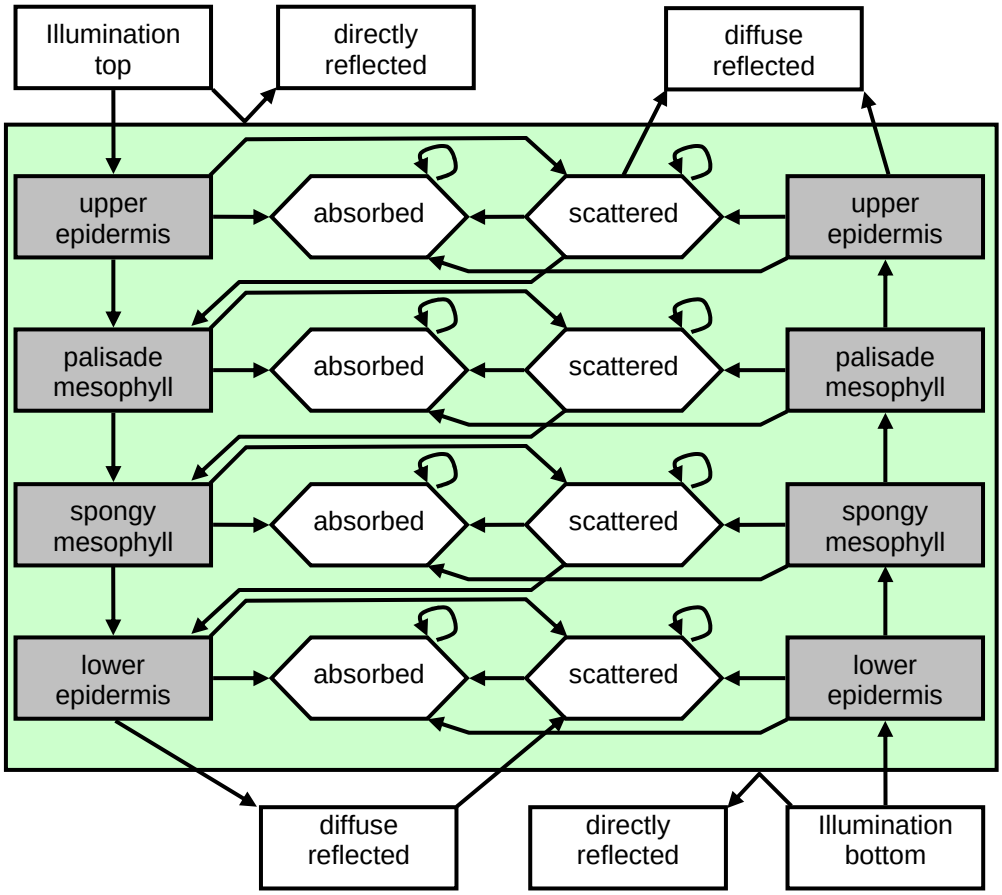


Figure 2.4. Stochastic radioactive transfer model of interactions between leaf structural components and electromagnetic radiation (EMR) (Jacquemoud and Ustin 2001)

# 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 1$^{st}$ spectral derivative method. Our results indicated that the 1$^{st}$ 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 1$^{st}$ 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 1$^{st}$ 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-10$P_2O_5$-20$K_2O$ 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 $\lambda_n$ 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.

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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)

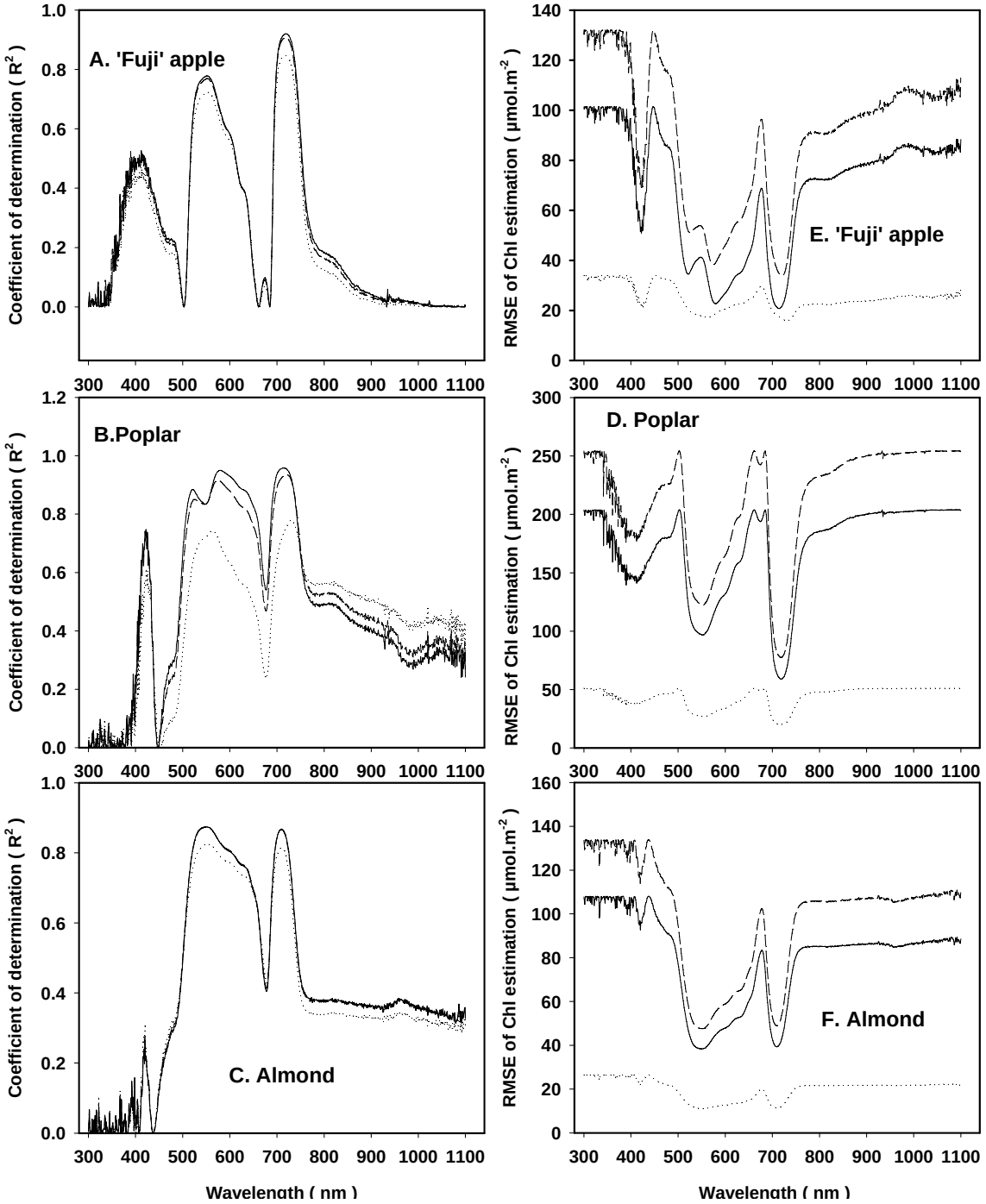


Figure 3.1 Coefficients of determination ($R^2$) and root mean square errors (RMSE) for the relationships between chlorophyll concentrations (Chl a, Chl b and Chl a+b) and reflectance values at 1 nm intervals from 300nm to 1100 nm in leaves of apple (A, D), poplar (B, E) and almond (C, F). Apple = *Malus domestica* 'Fuji'; Poplar = *Populus trichocarpa* x *P. deltoids*; Almond = *Prunus dulcis* 'Nonpareil'. $R^2$ and RMSE values in each graph represent means of 72 leaves.

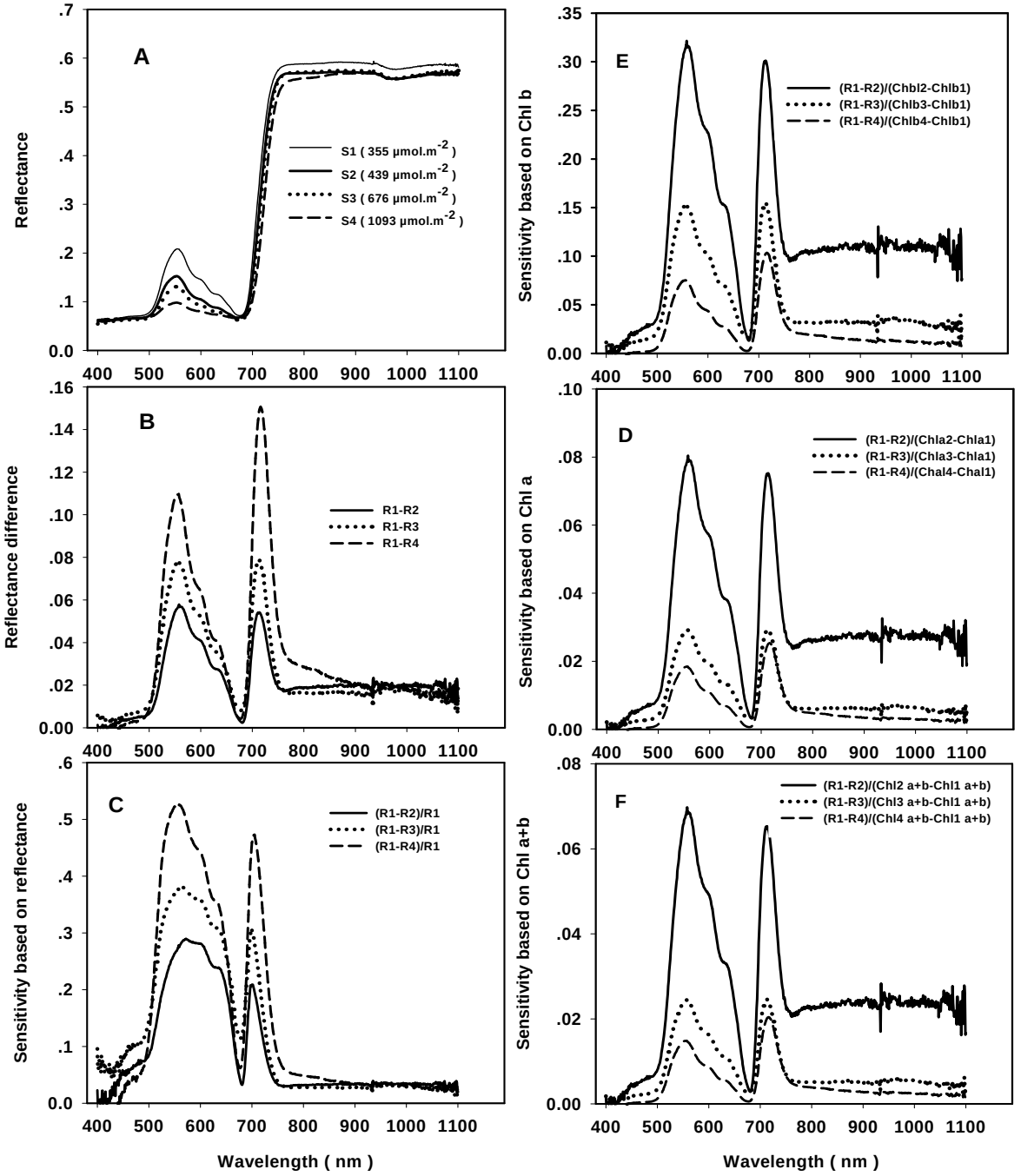


Figure 3.2 Original reflectance spectra (A), reflectance difference curves (B) and reflectance sensitivity curves (C-F) of four apple (*Malus domestica* 'Fuji') leaves (S1-S4) with different total chlorophyll concentrations (ChlT1-ChlT4). The curves of reflectance difference (B) were generated by subtracting the reflectance of S2, S3, and S4 (R2, R3, and R4) from the reflectance of S1 (R1). The sensitivity curves based on reflectance (C) were generated by dividing the reflectance difference value by R1. The sensitivity curves based on Chl a, Chl b and Chl a+b (D-F) were generated by dividing the reflectance difference value by the difference in concentrations of Chl a, Chl b and Chl a+b between leaf samples S2, S3, S4 and S1 then multiplying by 100.

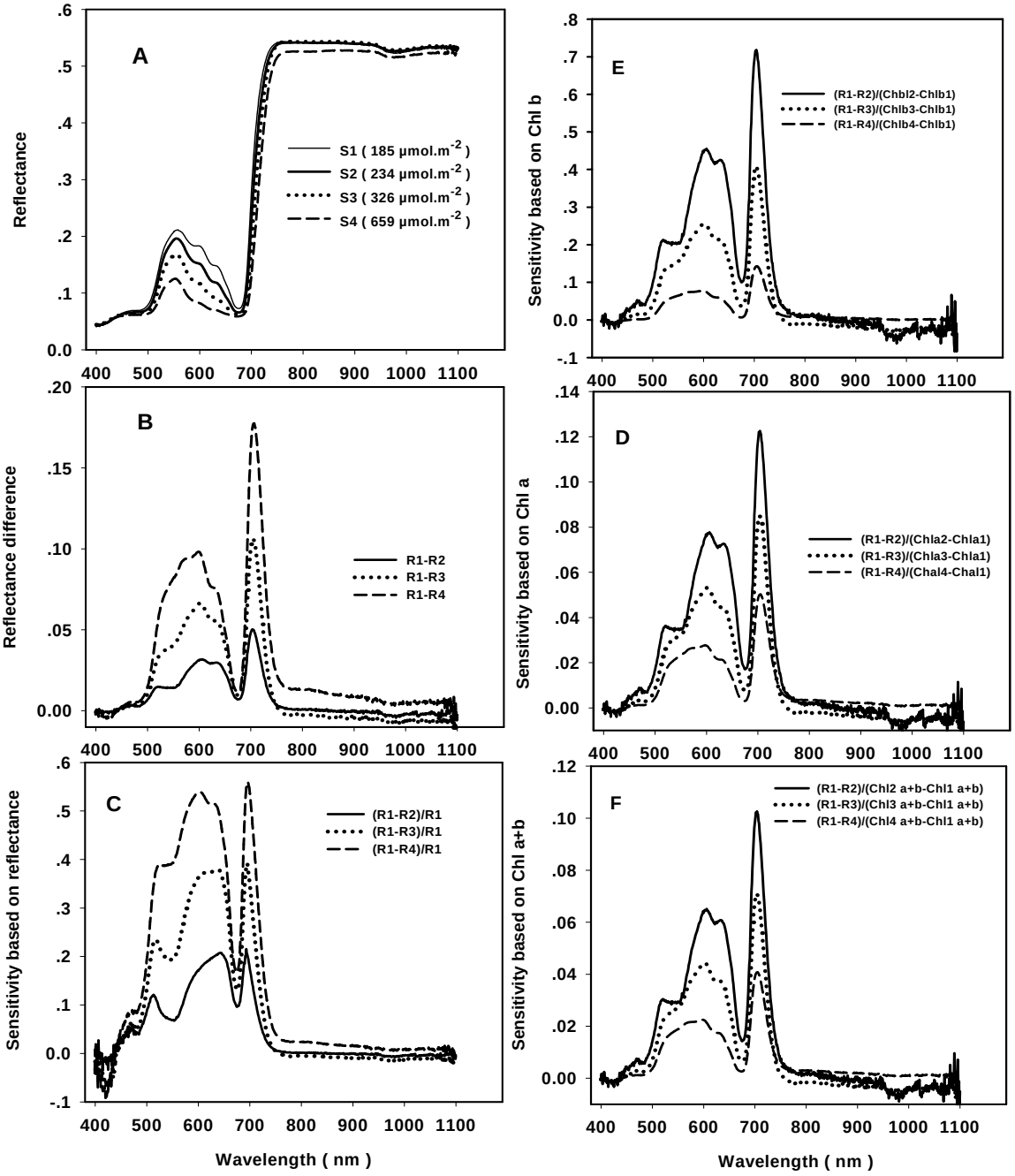


Figure 3.3 The original reflectance spectra (A), reflectance difference curves (B) and reflectance sensitivity curves (C-F) of four poplar (*Populus trichocarpa* x *P. deltoids*) leaves (S1-S4) with different total chlorophyll concentrations (ChlT1-ChlT4). The curves of reflectance difference (B) were generated by subtracting the reflectance of S2, S3, and S4 (R2, R3, and R4) from the reflectance of S1 (R1). The sensitivity curves (C) based on reflectance were generated by dividing the reflectance difference value by R1. The sensitivity curves based on Chl a, Chl b and Chl a+b (D-F) were generated by dividing the reflectance difference value by the difference in concentrations of Chl a, Chl b and Chl a+b between leaf sample S2, S3, S4 and S1 then multiple 100 (D-F).

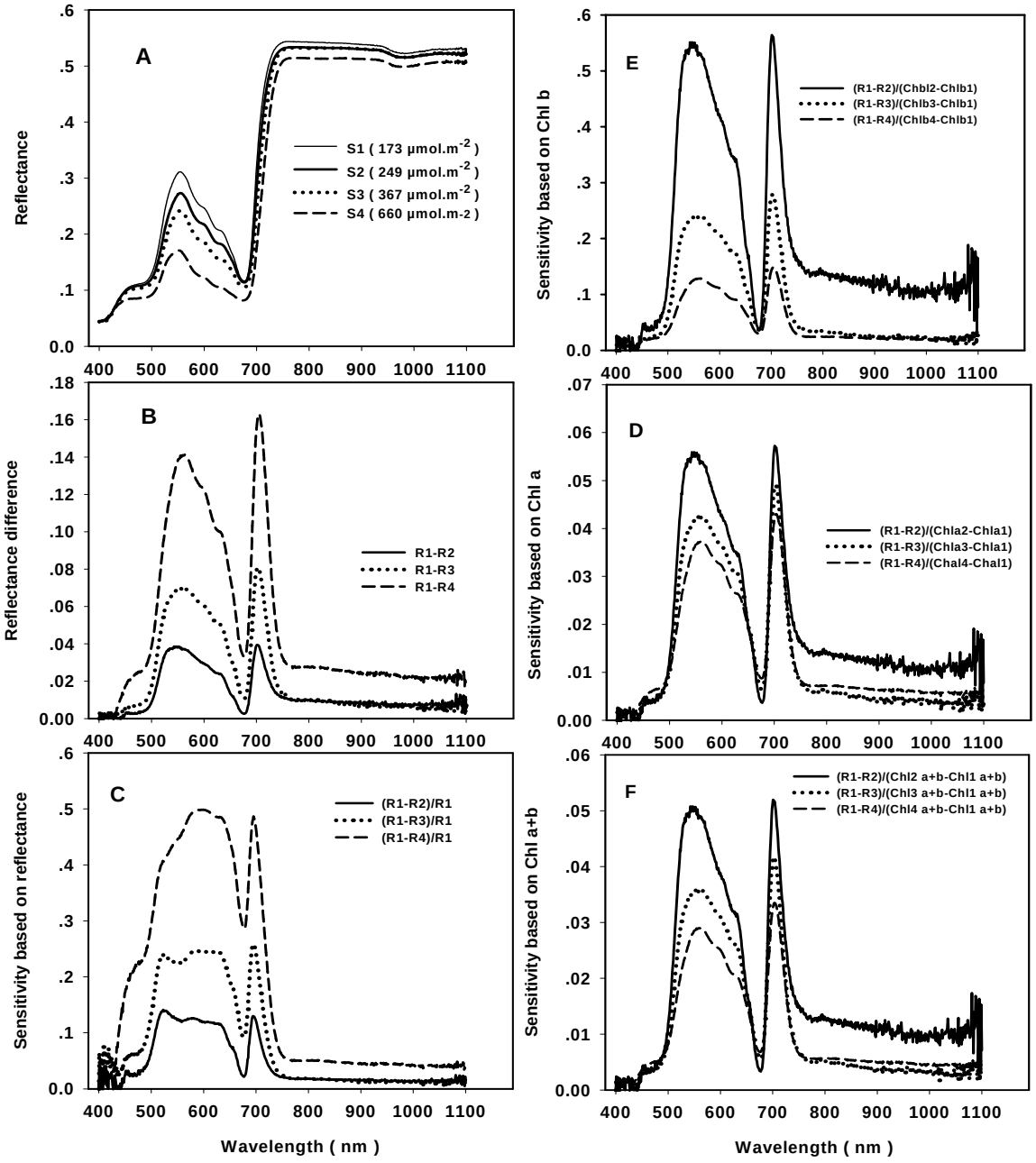


Figure 3.4 The original reflectance spectra (A), reflectance difference curves (B) and reflectance sensitivity curves (C-F) of four almond (*Prunus dulcis* 'Nonpareil') leaves (S1-S4) with different total chlorophyll concentrations (ChlT1-ChlT4). The curves of reflectance difference (B) were generated by subtracting the reflectance of S2, S3, and S4 (R2, R3, and R4) from the reflectance of S1 (R1). The sensitivity curves (C) based on reflectance were generated by dividing the reflectance difference value by R1. The sensitivity curves based on Chl a, Chl b and Chl a+b (D-F) were generated by dividing the reflectance difference value by the difference in concentrations of Chl a, Chl b and Chl a+b between leaf sample S2, S3, S4 and S1 then multiple 100 (D-F).

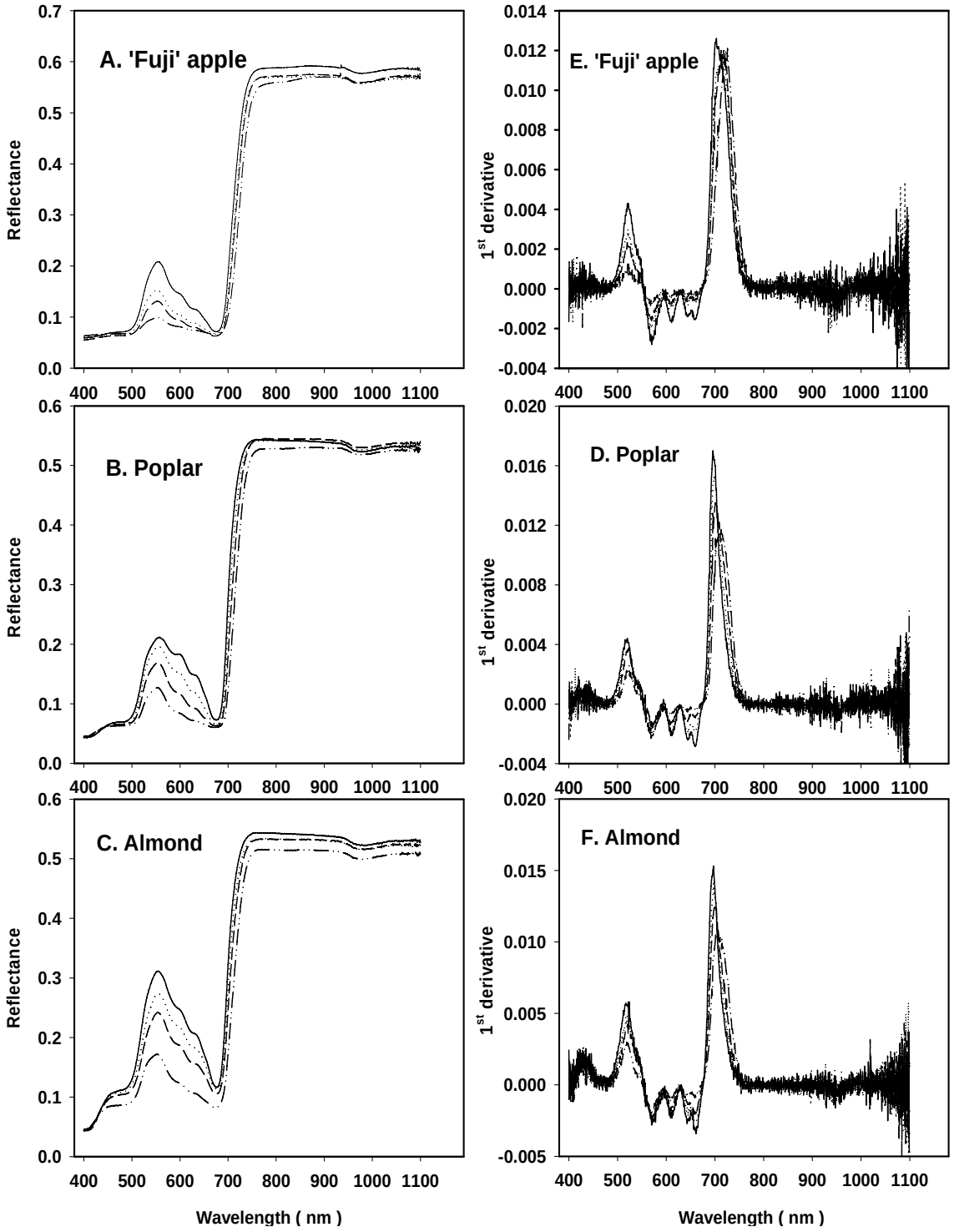


Figure 3.5 The original reflectance spectra (A-C) of four leaves (S1-S4) with different total chlorophyll concentrations and the corresponding the 1st derivative spectra (D-F) for the same leaves. Apple = *Malus domestica* 'Fuji'; Poplar = *Populus trichocarpa* x *P. deltoids*; Almond = *Prunus dulcis* 'Nonpareil'.

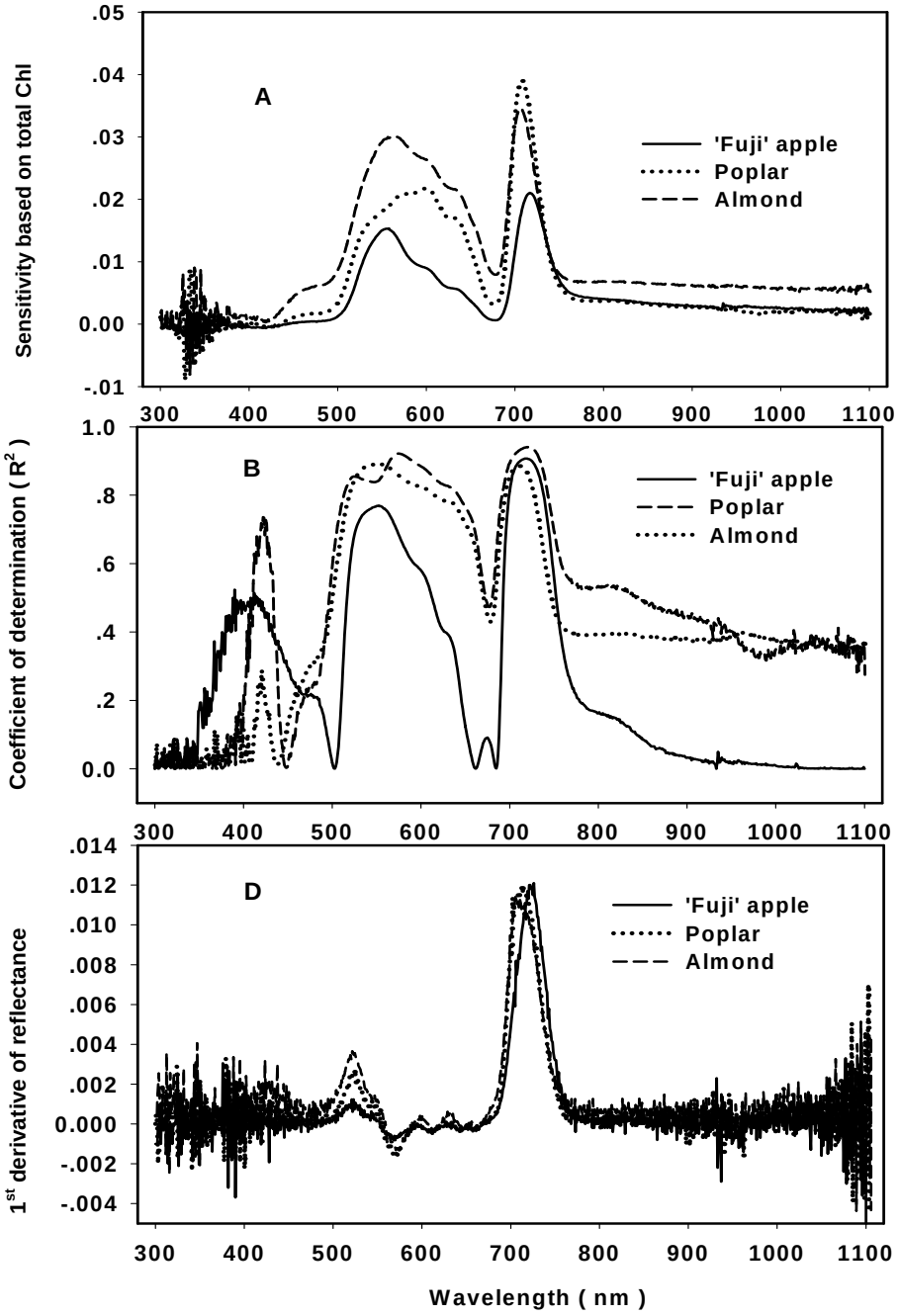


Figure 3.6 Comparison of peaks obtained from three methods used to select optimum wavelengths for assessing chlorophyll (Chl) concentrations in leaves of apple, poplar, and almond. (A) Reflectance sensitivity curves based on differences in total Chl generated by dividing the reflectance difference value with the difference in total Chl concentration between two leaves (Apple, 335 and 1093 µmol.m$^{-2}$; poplar,185 and 659 µm.m$^{-2}$; almond 173 and 660 µmol.m$^{-2}$). (B) Coefficient of determination ($R^2$) for the relationship between total Chl concentration and reflectance values at 1 nm intervals from 300nm to 1100 nm (n= 72 leaves per genotype); and (C) 1$^{st}$ derivative spectra developed by using the reflectance of the leaf with highest Chl concentration. Apple = *Malus domestica* 'Fuji'; Poplar = *Populus trichocarpa* x *P. deltoids*; Almond = *Prunus dulcis* 'Nonpareil'.

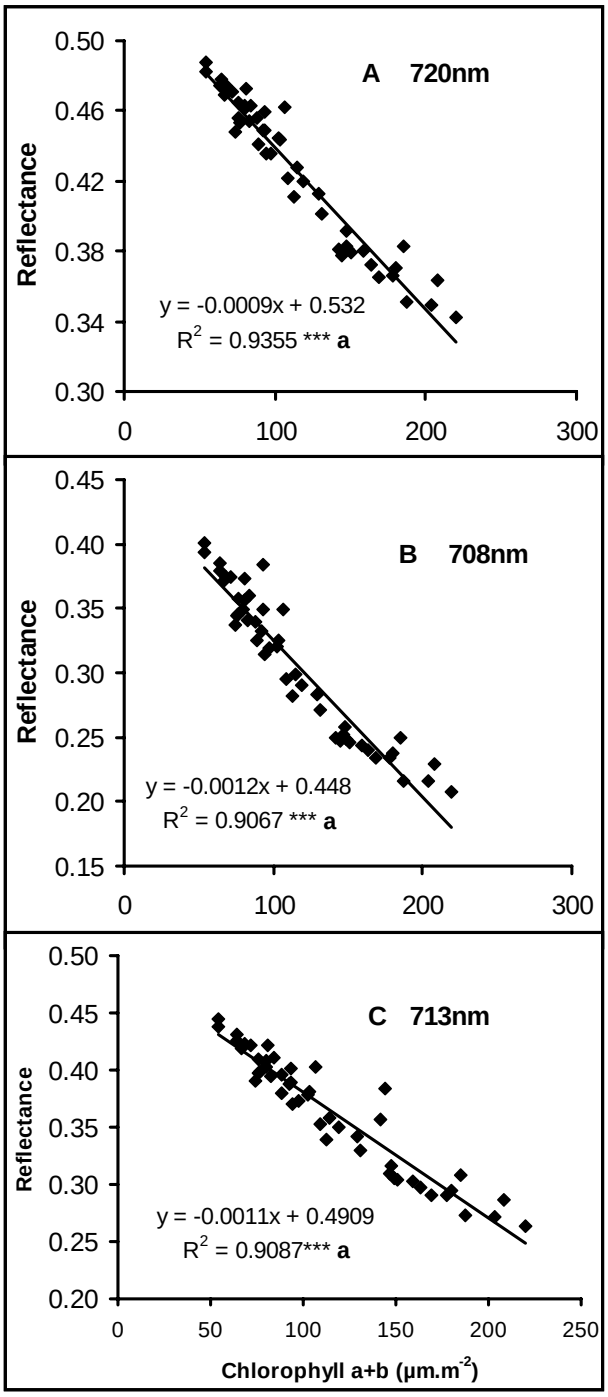


Figure 3.7 Relationship between total chlorophyll concentration (Chl a + b) in poplar (*P. trichocarpa* x *P. deltoids*) and reflectance values at optimum wavelengths selected for Chl assessment using three different methods. (A) 720 nm selected by using the coefficient of determination ($R^2$) for the relationship between total Chl concentration and reflectance values (B), 708 nm selected by using reflectance sensitivity analysis, and (C) 713 nm selected by using the 1st derivative method.*** $p<0.001$, $R^2$ followed by same letter are not significantly different ($p<0.05$, Fisher's Z-Test, n=48)

# CHAPTER 4

# OPTIMUM WAVELENGTH IDENTIFICATION AND INDICES EVALUATION FOR NONDESTRUCTIVE ASSESSMENT OF CHLOROPHYLL IN FRESH POPLAR LEAVES USING SPECTRAL REFLECTANCE

## 4.1 Abstract

One-year-old poplar (UCC-1, a hybrid of *Populus trichocarpa* × *P. deltoides*, Union Camp, Princeton, NJ) trees were grown with one of six different rates of nitrogen (N) fertilizer to produce leaves with different chlorophyll (Chl) concentrations (160 to 659μmol.m$^{-2}$) for identifying optimal wavelengths ($OW_{Chl}$) and evaluating indices for non-destructive Chl assessment using reflectance. Leaves with different Chl concentrations were scanned from 300 to 1100 nm, at 1 nm intervals, using a spectroradiometer to measure spectral reflectance. Concentrations of leaf Chl a, Chl b and Chl a+b were used to determine $OW_{Chl}$ usiing wavelength sensitivity analysis and simple linear regressions between reflectance values and Chl concentrations. The coefficient of determination ($R^2$) and root mean square error (RMSE) from the regression analysis and results of the wavelength sensitivity analysis indicated that wavebands from two regions of the spectrum, red edge (700 -730 nm) and visible (540 – 615 m), had larger $R^2$, higher wavelength sensitivity, and smaller RMSE than peaks at other wavebands. These wavebands were determined to be $OW_{Chl}$ for Chl (Chl a, Chl b and Chl a+b) assessment in poplar leaves.

The accuracy ($R^2$ and the corresponding RMSE) of different published indices compared in our study differed greatly and was lower than that of the indices developed with the $OW_{Chl}$ we determined using a combination of regression and wavelength

sensitivity. The main reason that the published indices were less accurate (smaller $R^2$ with larger RMSE) was because the Chl related wavelengths used in these indices were not close to the $OW_{Chl}$. Our results indicate that identification of $OW_{Chl}$ is very important for indices development. When the Chl-related wavelengths (675, 695, 698, 700, 705 or 710 nm) in different proposed indices were replaced by the $OW_{Chl}$ we determined in the visible (580, 563 and 574nm) or in red edge (715, 730 and 720nm) for Chl a, Chl b or Chl a+b, all results using modified indices were more accurate than the results using the original indices. The most accurate indices for Chl assessment of poplar leaves were determined to be $R_{750\text{-}1000}/R_{ow}$, $(R_{750\text{-}1000}-R_{ow})/(R_{750\text{-}1000}+R_{ow})$, $R_{430\text{-}490}/R_{ow}$ and $(R_{430\text{-}490}-R_{ow})/(R_{430\text{-}490}+R_{ow})$, for Chl a, Chl b or Chl a+b, respectively. Although $OW_{Chl}$ differ among species, cultivars and Chl (Chl a, Chl b and Chl a+b), our results indicated that wavelength from 700 - 730nm and 540 - 580nm regions of the spectrum can be used as the $OW_{Chl}$ for a variety of species. Indices that use a combination of the $OW_{Chl}$ with a reference wavelength (RW) at either 430 - 490nm or 750 - 1100nm can be used to eliminate the effect of leaf texture on Chl assessment and more widely applicable across species. These indices can be simplified as $R_{RW}/R_{OW}$ and $(R_{RW} - R_{OW})/(R_{RW}+R_{OW})$.

## 4.2 Introduction

The chlorophylls, Chl a and Chl b, are essential pigments for the conversion of light energy to stored chemical energy (Gitelson et al. 2003, Richardson et al. 2002) and their concentrations in leaves is a limiting factor to photosynthetic potential and primary production (Curran et al. 1990, Filella et al. 1995, Peñuelas et al. 1995a, Peñuelas and Filella 1998, Richardson et al. 2002). Leaf Chl concentrations are also important

indicators of plant nutrition and stresses (Carter and Knapp 2001, Filella et al. 1995, Hendry 1987, Moran et al. 2000, Peñuelas and Filella 1998)

Traditionally, leaf extraction with organic solvents and spectrophotometric determination in solution was required for assessing Chl concentrations in plant samples (Arnon1949, Lichtenthaler 1987). Recently, alternative, nondestructive optical methods, based on the absorbance and/or reflectance of light by intact leaves, have been developed (Adams et al. 1999, Curran et al. 1990, Datt 1999a, Gamon and Surfus 1999, Markwell et al. 1995). These optical methods are simple to use, fast and can be used in the field (Buschmann and Nagel 1993, Buschmann et al. 1994, Gitelson and Merzlyak 1994b, Gitelson et al. 1996a, 1996b, Markwell et al. 1995). Most prior research has focused on developing Chl-related indices (Adams et al.1999, Blackburn 1998, Curran et al. 1990; Datt 1998, Datt 1999a, Gamon and Surfus 1999, Gitelson and Merzlyak 1994, Gitelson and Merzlyak 1996, Gitelson et al. 1996a, Gitelson et al. 1996b). These indices have been developed for nondestructive Chl assessment in a variety of plants (Adams et al. 1999, Blackburn 1998, Curran et al. 1990, Datt 1998, Datt 1999a, Datt 1999b, Gamon and Surfus 1999, Gitelson and Merzlyak 1994, Gitelson and Merzlyak 1996, Gitelson et al. 1996a, Gitelson and Merzlyak 1996b) using one specific Chl-related wavelength (e.g. 550, 698, 692 or 695 nm) (Carter 1994, Jacquemoud and Baret 1990, Moran and Moran 1998, Thomas and Gausman 1977), or using one Chl-related wavelength in combination with Chl-insensitive wavelength in the form of wavelength ratio (e.g. R698/R760) or some specific algorithm (e.g. $(R_{760}-R_{675})/(R_{760}-R_{675})$) (Moran et al. 2000, Peñuelas et al. 1995b, Richardson et al. 2002).

In general, prior published indices for Chl assessment have rarely been tested using data from species or genotypes other than those used to develop the indices (Richardson et al. 2002), and there is no accurate index that can be used universally across genotypes. We believe the main reason that these indices are not applicable across different studies and among genotypes is that either the Chl-related wavelengths used for developing the indices are not the $OW_{Chl}$ for assessing Chl or the $OW_{Chl}$ used for developing indices varies between studies and genotypes. Our objective was to evaluate the importance of using $OW_{Chl}$ in the development of Chl-related reflectance indices.

## 4.3 Materials and methods

### *4.3.1 Plant materials*

In 2000 and 2001, 1-year-old poplar (UCC-1, a hybrid of *Populus trichocarpa* × *P. deltoides*, Union Camp, Princeton, NJ) 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-10$P_2O_5$-20$K_2O$ 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.

### *4.3.2 Reflectance spectra and Chl determination*

At the end of August, 12 fresh leaves from 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 to 1100 nm at 1 nm interval using Li-1800 spectroradiometer (Li-Cor Inc., Lincoln, NE). The reflectance spectrum ($R_\lambda$) for each scan was calculated as the leaf radiance at wavelength λ divided by the standard reflectance radiance at wavelength λ. Two scans were made per leaf disc and then averaged. After scanning, each leaf disc was cut into small 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 concentrations for different N fertigation treatments ranged from 160 to 659$\mu m.m^{-2}$.

### *4.3.3 Regression analyses of spectral reflectance and Chl data*

Using Microsoft Visual Basic 6.0 (Microsoft Corp., Redmond, WA), custom software was developed to directly perform simple linear regression equations (linear-least-squares-fit) and calculate root mean square error (RMSE) and coefficients of determination ($R^2$) between the spectral reflectance reading at each 1 nm wavelength interval from 300 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 optimum wavelengths for estimating Chl

concentrations using reflectance ($OW_{Chl}$). 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 optimum wavelengths for estimating Chl concentrations. Difference between $R^2$ was tested as pairwise comparison using Fisher's Z-test (Lawley 1938 ).

### *4.3.4 Wavelength sensitivity analysis*

Reflectance curves of four leaves as single leaf samples (S1, S2, S3 and S4) with different total Chl concentrations were selected for wavelength sensitivity analysis. The reflectance curves for wavelengths at 1 nm increments from 300 to 1100 nm were developed for poplar leaves with Chl concentrations of 185 (Chl1), 234 (Chl2), 337 (Chl3) and 659 (Chl4) $\mu mol.m^{-2}$. The reflectance values for leaves with Chl2 (R2), Chl3 (R3) and Chl4 (R4) were then subtracted from the reflectance value of the leaf with lowest Chl concentration (R1) at each measured wavelength. Differences in reflectance values (R1-R2; R1-R3; R1-R4) were used to generate reflectance difference curves (e.g. wavelength vs. reflectance difference at each wavelength). Sensitivity curves based on differences in reflectance were generated by dividing the reflectance difference value from reflectance difference curves by the reflectance of R1 [e.g. (R1-R2)/R1]. These resultant sensitivity values based on reflectance at each wavelength were then plotted against the wavelength at which each reflectance measurement was taken (e.g. wavelength vs. sensitivity). Sensitivity curves based on 100 $\mu mol.m^{-2}$ 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 with lowest Chl concentration (Chla1) and the Chl concentration in other leaves (Chla2, Chla3, Chla4) (e.g. [(R1-R2)/(Chla2-Chla1)]*100). The resultant sensitivity values based on 100 $\mu mol.m^{-2}$ 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.

### *4.3.5 Calculation of leaf reflectance indices*

Reflectance spectra were transformed using published indices that have been recommended as indicators of foliar Chl or greenness assessment in remote sensing (Table 4.1). These indices were developed as a simple ratio (SR), a normalized difference vegetation index (NDVI), photochemical reflectance index (PRI), or structure insensitive pigment indices (SIPI).

A SR is one of the most popular indices frequently used in remote sensing to assess the abundance and vigor of vegetation and calculated as the ratio of two single wavelengths. The SR is also called vegetation index (VI) if the ratio is between NIR and red wavelengths of the spectrum (Richardson et al. 2002). Some indices targeted directly at either Chl a or Chl b; therefore the SR is called pigment specific simple ratio for Chl a (PSSR a) and Chl b (PSSR b) (Blackburn 1998).

A NDVI is strongly correlated with leaf Chl concentration (Gamon et al. 1995a, Peñuelas and Filella 1998, Richardson et al. 2002) and is a standard index used in remote sensing (Gamon and Qiu 1999). NDVI is calculated as $(R_{NIR}-R_{red})/(R_{NIR}+R_{red})$; here $R_{NIR}$ is the reflectance in the NIR region of the spectrum and $R_{red}$ is the reflectance in the red

region. The most popular NDVI is calculated as NDVI = $(R_{750} - R_{675})/(R_{750} + R_{675})$; here $R_{750}$ is the reflectance at 750 nm and $R_{675}$ is the reflectance at 675 nm. The revised version of the NDVI is called Chl Normalized difference index (Chl NDI), which is more highly correlated with leaf Chl concentration and more sensitive to a wider range of Chl concentrations when calculated as Chl NDI = $(R_{750} - R_{705})/(R_{750} + R_{705})$ (Richardson et al. 2002, Gitelson and Merzlyak 1994b).

PRI is an index of xanthophyll cycle pigment activity (Gamon et al. 1997, Gamon and Surfus 1999, Peñuelas and Filella 1998). 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) (Filella et al. 1995, Gamon et al. 1992, Peñuelas et al. 1995). Over longer time spans, or across species or sites, PRI is positively correlated with photosystem (PS) II efficiency as measured by Chl fluorescence and the Chl:carotenoids ratio, which may itself be an indicator of photosynthetic efficiency (Sims and Gamon 2002).

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

## 4.4 Results and discussion

### *4.4.1 Optimum wavelength identification*

$R^2$-curves showed $R^2$ peaks in the ultraviolet (UV) (400-440nm), visible (530-650nm), and red edge regions (690-750nm) of the spectra (Figure 4.1B). Any of these peak-

related wavelengths could be used for Chl assessment; however, wavelengths with both larger $R^2$ and smaller RMSE (Figure 4.1C) had the highest accuracy for Chl assessment. The $R^2$ peaks in the red edge and visible regions for Chl a, Chl b, and Chl a+b were much larger than the $R^2$ peak in the UV region (similar results were reported in Chapter 3).

Many published report noticed the indices developed for one genotype could rarely be used across genotypes (Richardson et al. 2002). We found this phenomena also existed among different Chl (Chl a, Chl b and Chl a+b). The peak apexes and corresponding wavelengths differed among chlorophylls (e.g. Chl a, Chl b and Chl a+b) in both visible and red edge regions (Table 4.2). In order to accurately assess different Chl, the specific Chl related $OW_{Chl}$ should be selected for Chl assessment. The most accurate wavelengths for different Chl (e.g. Chl a, Chl b and Chl a+b) assessment are found in two narrow regions: visible (550 - 586 nm) and red edge (702– 745 nm) (Table 4.2). Therefore, a "common" $OW_{Chl}$ for different Chl from either the visible (565 -570 nm) or red edge (710 – 730 nm) regions can be used to accurately assess different Chl, although this procedure is not as accurate as using the specific $OW_{Chl}$ for different Chl. The $R^2$ and corresponding wavelength for Chl a+b was between the $R^2$ and the corresponding wavelength for Chl a and Chl b, but tended to be closer to the $R^2$ and the corresponding wavelength of Chl a (similar results were reported in Chapter 3). The proximity of $OW_{Chl}$ for total Chl to Chla is because about 80% total Chl is in the form of Chl a in plant leaves.

The original reflectance spectra of poplar leaves with different Chl concentrations showed only one peak in the visible region at 530-580 nm (Figure 4.1A). After the original reflectance values were transformed for wavelength sensitivity analysis, peaks were evident in both the red edge region (685-730 nm) and visible region (560-650 nm) of the spectra for

wavelength sensitivity curves of Chl a, Chlb and Chl a+b (Figure 4.1D-F). The peak in the red edge region of the wavelength sensitivity curve was much higher than the peak in the visible region. High $R^2$ in combination with high wavelength sensitivity indicated that the wavelength associated with peaks in both red edge and visible regions could be used as $OW_{Chl}$ (similar results are reported in Chapter 3). Many indices have been developed for Chl assessment (Curran et al. 1990, Gitelson and Merzlyak 1994a, Gitelson and Merzlyak 1996b, Gitelson et al. 1996a, Gitelson et al.1996b, Blackburn 1998, Datt 1998, Datt 1999a, Adams et al. 1999, Gamon and Surfus 1999). However, these proposed indices could not be used between different studies and plant species. One important reason is the importance of using $OW_{Chl}$ for indices development is not widely recognized and the Chl-related wavelengths used in the indices are not the $OW_{Chl}$.

### *4.4.2 Comparison of published indices used in Chl assessment*

There are many published indices that have been developed for vegetation remote sensing and nondestructive Chl assessment in a variety of plants (Adams et al. 1999, Blackburn 1998, Curran et al. 1990, Datt 1998, Datt 1999a, Datt 1999b, Gamon and Surfus 1999, Gitelson and Merzlyak 1994a, Gitelson and Merzlyak 1996, Gitelson et al. 1996a, Gitelson et al.1996b). Generally, published indices have been developed based on one or two Chl-related wavelengths and one Chl-unrelated wavelength (reference wavelength, RW). The Chl-related wavelengths used in prior published indices are usually the wavelengths in red to red edge (675– 710 nm), visible (530-570 nm) or blue (420-445 nm) regions of the spectrum. The RW is usually in the near infra red (NIR) (750 - 940 nm) and is closely related to leaf structure (Richardson et al. 2002).

The accuracy ($R^2$ and the corresponding RMSE) of the published indices compared in our study differed greatly. Among the 20 most popular published indices, our results showed that the indices $R_{750}/R_{700}$, $R_{850}/R_{710}$, $R_{698}/R_{760}$, $R_{710}/R_{760}$, $R_{695}/R_{760}$, $R_{695}/R_{420}$, $R_{675}/R_{700}$, $(R_{750}-R_{705})/(R_{750}+R_{705})$, $(R_{850}-R_{710})/(R_{850}-R_{680})$ and $(R_{800}-R_{700})/(R_{800}+R_{700})$ had larger $R^2$ and smaller RSME indicating they were more accurate for Chl a assessment than other indices (Table 4.3); the indices $R_{675}/R_{700}$, $R_{850}/R_{710}$, $R_{750}/R_{700}$, $R_{750}/R_{550}$, $(R_{850}-R_{710})/(R_{850}-R_{680})$ and $(R_{750}-R_{705})/(R_{750}+R_{705})$ had larger $R^2$ and smaller RSME indicating they were more accurate for Chl b assessment (Table 4.4); while the indices $R_{850}/R_{710}$, $R_{675}/R_{700}$, $R_{750}/R_{700}$, $R_{710}/R_{760}$, $(R_{750}-R_{705})/(R_{750}+R_{705})$, $(R_{850}-R_{710})/(R_{850}-R_{680})$ and $(R_{800}-R_{700})/(R_{800}+R_{700})$ were more accurate over other indices for total Chl (Chl a+b) assessment (Table 4.5). For example, the $R_{750}/R_{700}$ (Gitelson et al. 1996a) and $(R_{750}-R_{705})/(R_{750}+R_{705})$ (Gitelson and Merzlyak 1994b, Gitelson 1994a, Gamon and Surfus 1999) had greater accuracy (larger $R^2$ and smaller RMSE) for Chl a, Chl b and Chl a+b assessment than the $R_{800}/R_{650}$ (Blackburn 1998) and $(R_{750}-R_{675})/(R_{750}+R_{675})$ (Gamon and Qiu 1999, Richardson and Berlyn 2002) (Table 4.3, Table 4.4, Table 4.5).

Using the same or similar wavelengths two wavelength, SR indices were better than NDVI and other indices. Moreover, the published indices were more accurate when they included two wavelengths: one RW in the NIR region (750 to 850 nm) and the other a Chl-related wavelength (420, 675, 695, 698, 700, 705 or 710 nm). The reason that published indices ($R_{695}/R_{760}$, $R_{940}/R_{675}$, $R_{800}/R_{650}$, $(R_{800}-R_{680})/(R_{800}+R_{680})$, $(R_{750}-R_{675})/(R_{750}+R_{675})$, $(R_{750}-R_{680})/(R_{750}+R_{690})$ are less accurate (smaller $R^2$ with larger RMSE, Table 4.3, Table 4.4 and Table 4.5) is because the Chl related wavelengths (650nm 675

nm, 695nm, 680 nm and 690 nm) used in these indices are not close to the $OW_{Chl}$. The closer the Chl-related wavelength in the index was to the $OW_{Chl}$ we identified for assessment of Chl a (715 nm), Chl b (730 nm) or Chl a+b (720nm), the more accurate the index for Chl a, Chl b or Chl a+b assessment.

Identification of $OW_{Chl}$ is very important for developing optimum indices with $OW_{Chl}$ for Chl assessment. When the Chl-related wavelengths of 675, 695, 698, 700, 705 or 710 nm from published indices (Table 4.1) were replaced by the $OW_{Chl}$ we determined for Chl a (715 nm), Chl b (730 nm) or Chl a+b (720nm) and the RW was not changed from the published indices, all the modified indices were more accurate (had larger $R^2$ and smaller RSME) than the corresponding published indices (Compare Table 4.3, Table 4.4 and Table 4.5 with Table 4.6). However, the importance of using $OW_{Chl}$ for indices development is not widely recognized. That is why so most published reports mainly focus on indices development and evaluation rather than identifying the $OW_{Chl}$ and developing the optimum indices with the $OW_{Chl}$ for Chl assessment (Carter 1994, Elvidge and Chen 1995, Gamon and Surfus 1999, Moran et al. 2000, Richardson and Berlyn 2002, Richardson et al. 2002). Our results indicated that, by using the $OW_{Chl}$, the indices with only one Chl-related $OW_{Chl}$ was still more accurate for assessing Chl than using any indices without $OW_{Chl}$. The accuracy of indices is further improved when $OW_{Chl}$ was combined with one RW to compensate the effect of leaf texture (Table 4.6).

### *4.4.3 Optimum indices determination*

Based on $R^2$ and wavelength sensitivity analysis the OW for assessment of Chl a, Chl b and Chl a+b were determined to be 580, 563 and 575 nm in the visible region and

715, 730 and 720 nm in the red edge region, respectively (Table 4.2). The indices for Chl assessment were developed by incorporating the identified $OW_{Chl}$ for Chl a, Chl b and Chl a+b with each wavelength from 300 to 1100nm in the form of an SR ($R_{300\text{-}1100}/R_{580}$, $R_{300\text{-}1100}/R_{563}$, $R_{300\text{-}1100}/R_{575}$, $R_{300\text{-}1100}/R_{715}$, $R_{300\text{-}1100}/R_{730}$, or $R_{300\text{-}1100}/R_{720}$), a NDVI [$(R_{300\text{-}1100}-R_{580})/(R_{300\text{-}1100}+R_{580})$, $(R_{300\text{-}1100}-R_{563})/(R_{300\text{-}1100}+R_{563})$, $(R_{300\text{-}1100}-R_{575})/(R_{300\text{-}1100}+R_{575})$], or a Chl NDI [$(R_{300\text{-}1100}-R_{715})/(R_{300\text{-}1100}+R_{715})$, $(R_{300\text{-}1100}-R_{730})/(R_{300\text{-}1100}+R_{730})$, or $(R_{300\text{-}1100}-R_{720})/(R_{300\text{-}1100}+R_{720})$]. These indices resulted in $R^2$ curves for Chl a, Chl b or Chl a+b with three peaks: one in the NIR region (750-1100 nm), one in the blue region (350-550 nm), and another in the visible to red region (550-650nm) (Figure 4.2, Figure 4.3). The peaks of the $R^2$-curves in the NIR and blue regions were much larger than those in the visible region.

The peaks on the RMSE-curves were inverted compared to peaks on the $R^2$-curves, and peaks with the largest $R^2$ values had the smallest RMSE (Figure 4.2, Figure 4.3), validating that $R^2$ was a reliable parameter for selecting the optimum indices for Chl assessment. The indices using SR or NDVI that incorporated one RW in the NIR region (750 to 1000 nm) or one blue region (400 to 490 nm) with the $OW_{Chl}$ in red edge (715, 730 and 720 nm for Chl a, Chl b and Chl a+b, respectively) or visible (580, 563 and 574 nm for Chl a, Chl b and Chl a+b, respectively) had the largest $R^2$ and smallest RMSE. In general, the indices that used one RW in the NIR region and one $OW_{Chl}$ produced $R^2$-curves with wider and more stable curves than indices that used one RW in the blue region with one $OW_{Chl}$ (Figure 4.2, Figure 4.3).

The optimum indices for Chl assessment varied with Chl type. Chl a or Chl a+b was best assessed using indices that incorporated one RW in the NIR region with the

$OW_{Chl\ a}$ (715 nm) or $OW_{Chl\ a+b}$ (720nm); whereas Chl b was best assessed using indices that incorporated one RW in the blue region (430 - 490nm) with the $OW_{Chl\ b}$ (563 nm) (Figure 4.2, Figure 4.3). Accuracy (larger $R^2$ and smaller RMSE) of indices using just a single wavelength either in the visible or red edge region could be improved by incorporating the $OW_{Chl}$ with one RW either from 400 to 490nm or 750 to 1000nm as an SR or NDVI (Figure 4.2, Figure 4.3, Table 4.6, Table 4.7). Most published indices use one Chl-insensitive wavelength from 750-940 nm as the RW (Gamon and Surfus 1999, Blackburn 1998, Carter 1993, Carter 1994, Chappelle et al. 1992, Datt 1999a, Gamon and Qiu 1999, Gamon et al. 1992, Gamon et al. 1997, Gietelson and Merzlyak 1994a, Gietelson and Merzlyak 1994b, Gitelson et al. 1996a, Moran et al. 2000, Peñuelas et al. 1995, Richardson and Berlyn 2002, Richardson et al.2002). Our results showed that both the regions in blue (400 - 490nm) and NIR (750-1100 nm) can be used as the RW in combination with the Chl-related $OW_{Chl}$ for indices development to assess Chl (Figure 4.2, Figure 4.3).

The most useful indices would allow for accurate Chl assessment across a wide range of genotypes (Richardson et al. 2002). Although we found that the $OW_{Chl}$ differed among genotypes (e.g. apple, almond, poplar, purple leaf plum and purple leaf flowering cherry in Table 5-3) and Chl type (Chl a, Chl b and Chl a+b), our results indicate that there are two regions of $OW_{Chl}$ (700 – 730 nm and 540 – 580 nm) and two regions of RW (430 – 490 nm and 750 – 1100 nm) that are narrow enough for developing indices for specific genotypes or Chl types, and wide enough for developing indices that have potential to be consistent across genotypes and Chl types. These optimal indices that meet these criteria were determined to be $R_{RW}/R_{OW}$, or $(R_{RW}-R_{OW})/(R_{RW}+R_{OW})$.

## 4.5 Conclusions

Our results verified the importance of $OW_{Chl}$ utilization in indices development for Chl assessment. Indices that only had one Chl-related $OW_{Chl}$ were still more accurate than the indices that did not use $OW_{Chl}$. Indices accuracy was further improved when $OW_{Chl}$ (700-730 nm or 540-580 nm) in combination with one RW either in blue (430 - 490nm) or NIR (750-1100 nm) in indices development. These optimal indices developed with $OW_{Chl}$ and RW can be used across genotypes and Chl types, and can be summarized $R_{RW}/R_{OW}$, or $(R_{RW}-R_{OW})/(R_{RW}+R_{OW})$.

## 4.6 References

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Table 4.1 Published indices used for leaf-level or canopy-level chlorophyll (Chl) assessment in remote sensing

| Indices[z] | Name[y] | Reference |
|---|---|---|
| $R_{750}/R_{700}$ | VI | Gitelson et al. 1996a |
| $R_{750}/R_{550}$ | VI | Gitelson et al. 1996a |
| $R_{850}/R_{710}$ | VI | Datt 1999a |
| $R_{710}/R_{760}$ | VI | Carter 1993,1994 |
| $R_{695}/R_{760}$ | VI | Carter 1993,1994 |
| $R_{605}/R_{760}$ | VI | Carter 1993,1994 |
| $R_{695}/R_{420}$ | VI | Carter 1993,1994 |
| $R_{675}/R_{700}$ | VI | Chappelle et al 1992 |
| $R_{940}/R_{675}$ | VI | Carter 1994 |
| $R_{800}/R_{680}$ | VI | Blackburn 1999 |
| Chl a: $R_{800}/R_{675}$ | PSSR a | Blackburn 1998 |
| Chl b: $R_{800}/R_{650}$ | PSSR b | Blackburn, 1998 |
| $(R_{800}-R_{445})/(R_{800}-R_{680})$ | SIPI | Moran et al. 2000, Peñuelas et al. 1995 |
| $(R_{850}-R_{710})/(R_{850}-R_{680})$ | NDVI | Datt, 1999 a |
| $(R_{800}-R_{700})/(R_{800}+R_{700})$ | NDVI | Gietelson & Merzlyak 1994 a and b |
| $(R_{800}-R_{680})/(R_{800}+R_{680})$ | NDVI | Blackburn 1998 |
| $(R_{750}-R_{675})/(R_{750}+R_{675})$ | NDVI | Gamon & Qiu 1999, Richardson & Berlyn 2002 |
| $(R_{531}–R_{570})/(R_{531}+R_{570})$ | PRI | Gamon et al. 1992, 1997, Peñuelas et al. 1995 |
| $(R_{750}-R_{680})/(R_{750}+R_{690})$ | NDVI | Richardson et al.2002 |
| $(R_{750}-R_{705})/(R_{750}+R_{705})$ | Chl NDI | Gitelson & Merzlyak 1994a, Gamon & Surfus 1999 |

[z]Indices used in index development. $R_x$ = reflectance at wavelength x (in nm)

[y]VI (vegetation index or simple ratio), pigment specific simple ratio for Chl a (PSSR a) or Chl b (PSSR b), normalized difference vegetation index (NDVI), Chl Normalized difference index (Chl NDI), photochemical reflectance index (PRI), and structure insensitive pigment index (SIPI).

Table 4.2 Peak range and optimum wavelengths ($OW_{Chl}$) for assessment of different chlorophyll (Chl) types (Chl a, Chl b and Chl a+b) in poplar leaves

| Chl type | Parameter[z] | Visible region | | | NIR region | | |
|---|---|---|---|---|---|---|---|
| | | Wavelength (nm) | $R^{2y}$ | RMSE [x] ($\mu mol.m^{-2}$) | Wavelength (nm) | $R^{2\,y}$ | RMSE [x] ($\mu mol.m^{-2}$) |
| Chl a | Peak range | 565-615 | 0.903-0.949 | 31.46-25.82 | 700-730 | 0.924-0.958 | 23.82-28.91 |
| | $OW_{Chl}$ | 580 | $0.9497^{***}$b | 25.82 | 715 | $0.9579^{***}$b | 23.82 |
| Chl b | Peak range | 550-570 | 0.724-0.744 | 14.72-15.13 | 710-745 | 0.711-0.780 | 13.98-15.38 |
| | $OW_{Chl}$ | 563 | $0.7440^{**}$a | 14.72 | 730 | $0.7801^{**}$a | 13.98 |
| Chl a + b | Peak range | 566-586 | 0.901-0.917 | 36.30-37.77 | 702-733 | 0.900-0.935 | 33.48-37.85 |
| | $OW_{Chl}$ | 575 | $0.9166^{***}$b | 36.30 | 720 | $0.9352^{***}$b | 33.48 |

[z]Peak range = wavelength range with larger $R^2$ and smaller RMSE for Chl assessment, $OW_{Chl}$ = optimal wavelength with largest $R^2$ and smallest RMSE for Chl assessment.

[y]Coefficient of determination ($R^2$) values from simple regression between reflectance values within peak range wavelengths or $OW_{Chl}$ and Chl concentrations within leaves, **p<0.01, ***p<0.001; $R^2$ followed by the same letter within a column are not significantly different (p<0.05, Fisher's Z-Test, n=72)

[x]Root mean square error (RMSE) from simple regression between reflectance values within Peak range wavelengths or $OW_{Chl}$ and Chl concentrations within leaves.

Table 4.3 The accuracy of using published indices and calibration equations for assessing chlorophyll a (Chl a) in poplar leaves

| Indices[z] | Name[y] | Calibration equation[x] | $R^{2w}$ | RSME [w] ($\mu mol.m^{-2}$) |
|---|---|---|---|---|
| $R_{750}/R_{700}$ | VI | $y = 1.7053x^2 + 40.872x - 33.936$ | $0.9664e^{***}$ | 23.77 |
| $R_{850}/R_{710}$ | VI | $y = -6.8891x^2 + 142.52x - 126.25$ | $0.9650e^{***}$ | 23.94 |
| $R_{698}/R_{760}$ | VI | $y = 1346.5x^2 - 1307.3x + 368.69$ | $0.9617e^{***}$ | 24.35 |
| $R_{710}/R_{760}$ | VI | $y = 502.89x^2 - 952.62x + 483.03$ | $0.9552e^{***}$ | 25.15 |
| $R_{695}/R_{760}$ | VI | $y = 2546.8x^2 - 1876.8x + 398.69$ | $0.9537e^{***}$ | 25.33 |
| $R_{695}/R_{420}$ | VI | $y = 13.55x^2 - 121.25x + 323.38$ | $0.9523e^{***}$ | 25.51 |
| $R_{675}/R_{700}$ | VI | $y = -751.33x^2 + 1012.5x - 159.87$ | $0.9478de^{***}$ | 26.06 |
| $R_{605}/R_{760}$ | VI | $y = 2745.3x^2 - 1927.6x + 390.64$ | $0.9132cd^{***}$ | 30.22 |
| $R_{800}/R_{675}$ | PSSR a | $y = 6.3458x^2 - 51.623x + 157.69$ | $0.9076c^{***}$ | 30.89 |
| $R_{750}/R_{550}$ | VI | $y = 1.5579x^2 + 70.142x - 138.63$ | $0.8236b^{**}$ | 40.50 |
| $R_{940}/R_{675}$ | VI | $y = -15.16x^2 + 286.56x - 1230.1$ | $0.4208a^{ns}$ | 77.53 |
| $R_{800}/R_{650}$ | PSSR b | $y = -12.87x^2 + 247.19x - 1058.6$ | $0.4176a^{ns}$ | 77.77 |
| $(R_{750}-R_{705})/(R_{750}+R_{705})$ | Chl NDI | $y = 546.92x^2 + 9.3931x + 25.205$ | $0.9579e^{***}$ | 24.82 |
| $(R_{850}-R_{710})/(R_{850}-R_{680})$ | NDVI | $y = 353.06x^2 - 13.719x + 25.754$ | $0.9577e^{***}$ | 24.84 |
| $(R_{800}-R_{700})/(R_{800}+R_{700})$ | NDVI | $y = 692.07x^2 - 282.98x + 71.902$ | $0.9474de^{***}$ | 26.10 |
| $(R_{531}-R_{570})/(R_{531}+R_{570})$ | PRI | $y = 9131.5x^2 + 1618.1x + 125.07$ | $0.8371b^{**}$ | 39.00 |
| $(R_{800}-R_{445})/(R_{800}-R_{680})$ | NDVI | $y = 130858x^2 - 269038x + 1383$ | $0.8275b^{**}$ | 40.07 |
| $(R_{800}-R_{680})/(R_{800}+R_{680})$ | NDVI | $y = -8525.1x^2 + 14912x - 6346.1$ | $0.4145a^{ns}$ | 77.99 |
| $(R_{750}-R_{675})/(R_{750}+R_{675})$ | NDVI | $y = -24484x^2 + 39491x - 15804$ | $0.3643a^{ns}$ | 81.53 |
| $(R_{750}-R_{680})/(R_{750}+R_{690})$ | NDVI | $y = -24484x^2 + 39491x - 15804$ | $0.3643a^{ns}$ | 81.53 |

[z]Indices used in index development. $R_x$ = reflectance at wavelength x (in nm)

[y]VI (vegetation index or simple ratio), pigment specific simple ratio for Chl a (PSSR a) or Chl b (PSSR b), normalized difference vegetation index (NDVI), Chl Normalized difference index (Chl NDI), photochemical reflectance index (PRI), and structure insensitive pigment index (SIPI).

[x]Calibration equation for converting algorithm transformed result to Chl a (y).

[w]Coefficient of determination ($R^2$) and Root mean square error (RMSE) values from regression of quadratic polynomial between leaf Chl a concentration and reflectance value transformation with different proposed indices, ns – not significant ($p>0.05$), **$p<0.01$, ***$p<0.01$; $R^2$ followed by the same letter are not significantly different ($p<0.05$, Fisher's Z-Test, n=72)

Table 4.4 The accuracy of using published indices and calibration equations for assessing chlorophyll b (Chl b) in poplar leaves

| Indices[z] | Name[y] | Calibration equation[x] | $R^{2w}$ | RSME [w] ($\mu mol.m^{-2}$) |
|---|---|---|---|---|
| $R_{675}/R_{700}$ | VI | $y = 677.02x^2 - 348.64x + 57.178$ | 0.8292f*** | 12.93 |
| $R_{850}/R_{710}$ | VI | $y = 50.192x^2 - 140.89x + 111.23$ | 0.7757ef** | 14.07 |
| $R_{750}/R_{700}$ | VI | $y = 10.151x^2 - 44.357x + 60.758$ | 0.7549def** | 14.50 |
| $R_{750}/R_{550}$ | VI | $y = 17.632x^2 - 88.406x + 122.36$ | 0.7457def** | 14.69 |
| $R_{710}/R_{760}$ | VI | $y = 576.01x^2 - 791.77x + 283.67$ | 0.6990bcde** | 15.63 |
| $R_{698}/R_{760}$ | VI | $y = 891.09x^2 - 702.81x + 149.26$ | 0.6098bcd* | 17.33 |
| $R_{605}/R_{760}$ | VI | $y = 1804.1x^2 - 1042.2x + 161.11$ | 0.6007bc* | 17.50 |
| $R_{695}/R_{420}$ | VI | $y = 8.1571x^2 - 59.672x + 119.63$ | 0.5875bc* | 17.74 |
| $R_{695}/R_{760}$ | VI | $y = 1452.6x^2 - 911.21x + 153.15$ | 0.5683bc* | 18.08 |
| $R_{800}/R_{675}$ | PSSR a | $y = 3.8595x^2 - 40.967x + 119.82$ | 0.5396b* | 18.59 |
| $R_{940}/R_{675}$ | VI | $y = -5.6063x^2 + 98.264x - 406.52$ | 0.1508a ns | 23.91 |
| $R_{800}/R_{650}$ | PSSR b | $y = -5.156x^2 + 89.886x - 367.72$ | 0.1450a ns | 23.96 |
| $(R_{850}-R_{710})/(R_{850}-R_{680})$ | NDVI | $y = 445.18x^2 - 317.88x + 68.406$ | 0.7085cde** | 15.44 |
| $(R_{750}-R_{705})/(R_{750}+R_{705})$ | Chl NDI | $y = 695.56x^2 - 368.18x + 60.419$ | 0.7076cde** | 15.46 |
| $(R_{800}-R_{700})/(R_{800}+R_{700})$ | NDVI | $y = 639.4x^2 - 491.88x + 105.89$ | 0.6691bcde** | 16.21 |
| $(R_{531}-R_{570})/(R_{531}+R_{570})$ | PRI | $y = 4435.2x^2 + 586.71x + 29.785$ | 0.6260bcd* | 17.03 |
| $(R_{800}-R_{445})/(R_{800}-R_{680})$ | NDVI | $y = 51774x^2 - 105981x + 54245$ | 0.5403b* | 18.58 |
| $(R_{800}-R_{680})/(R_{800}+R_{680})$ | NDVI | $y = -5170.3x^2 + 8329.7x - 3329.9$ | 0.1403ans | 23.99 |
| $(R_{750}-R_{675})/(R_{750}+R_{675})$ | NDVI | $y = -11067x^2 + 17391x - 6809.2$ | 0.1226a ns | 24.11 |
| $(R_{750}-R_{680})/(R_{750}+R_{690})$ | NDVI | $y = -11067x^2 + 17391x - 6809.2$ | 0.1226a ns | 24.11 |

[z]Indices used in index development. $R_x$ = reflectance at wavelength x (in nm)

[y]VI (vegetation index or simple ratio), pigment specific simple ratio for Chl a (PSSR a) or Chl b (PSSR b), normalized difference vegetation index (NDVI), Chl Normalized difference index (Chl NDI), photochemical reflectance index (PRI), and structure insensitive pigment index (SIPI).

[x]Calibration equation for converting algorithm result to Chl b (y)

[w]Coefficient of determination ($R^2$) and Root mean square error (RMSE) values from regression of quadratic polynomial between leaf Chl b concentration and reflectance value transformation with different proposed indices, ns – not significant ($p>0.05$), *$p<0.05$, **$p<0.01$; $R^2$ followed by the same letter are not significantly different ($p<0.05$, Fisher's Z-Test, n=72)

Table 4.5 The accuracy of using published indices and calibration equations for assessing chlorophyll a+b (Chl a+b) in poplar leaves

| Indices[z] | Name[y] | Calibration equation[x] | $R^{2w}$ | RSME [w] ($\mu mol.m^{-2}$) |
|---|---|---|---|---|
| $R_{850}/R_{710}$ | VI | $y = 43.303x^2 + 1.6257x - 15.024$ | 0.9504h*** | 33.03 |
| $R_{675}/R_{700}$ | VI | $y = -74.309x^2 + 663.83x - 102.7$ | 0.9499h*** | 33.08 |
| $R_{750}/R_{700}$ | VI | $y = 11.856x^2 - 3.4853x + 26.822$ | 0.9459h*** | 33.47 |
| $R_{710}/R_{760}$ | VI | $y = 1078.9x^2 - 1744.4x + 766.7$ | 0.9188fg*** | 36.09 |
| $R_{698}/R_{760}$ | VI | $y = 2237.5x^2 - 2010.1x + 517.94$ | 0.8648def** | 41.10 |
| $R_{800}/R_{675}$ | PSSR a | $y = 10.205x^2 - 92.59x + 277.51$ | 0.8637cdef** | 41.20 |
| $R_{605}/R_{760}$ | VI | $y = 4549.3x^2 - 2969.8x + 551.76$ | 0.8582bcde** | 41.69 |
| $R_{695}/R_{420}$ | VI | $y = 21.707x^2 - 180.92x + 443.01$ | 0.8449bcde** | 42.87 |
| $R_{750}/R_{550}$ | VI | $y = 19.19x^2 - 18.264x - 16.273$ | 0.8361bcd** | 43.65 |
| $R_{695}/R_{760}$ | VI | $y = 3999.3x^2 - 2788x + 551.84$ | 0.8324bcd** | 43.97 |
| $R_{940}/R_{675}$ | VI | $y = -20.766x^2 + 384.82x - 1636.6$ | 0.3512a ns | 76.45 |
| $R_{800}/R_{650}$ | PSSR b | $y = -18.025x^2 + 337.08x - 1426.3$ | 0.3465a ns | 76.67 |
| $(R_{750}-R_{705})/(R_{750}+R_{705})$ | Chl NDI | $y = 1242.5x^2 - 358.79x + 85.623$ | 0.9247gh*** | 35.53 |
| $(R_{850}-R_{710})/(R_{850}-R_{680})$ | NDVI | $y = 798.25x^2 - 331.6x + 94.161$ | 0.9236gh*** | 35.63 |
| $(R_{800}-R_{700})/(R_{800}+R_{700})$ | NDVI | $y = 1331.5x^2 - 774.85x + 177.79$ | 0.9041efg*** | 37.48 |
| $(R_{800}-R_{445})/(R_{800}-R_{680})$ | NDVI | $y = 182632x^2 - 375018x + 19257$ | 0.7718bc** | 49.10 |
| $(R_{531}-R_{570})/(R_{531}+R_{570})$ | PRI | $y = 13567x^2 + 2204.9x + 154.86$ | 0.7652b** | 49.64 |
| $(R_{800}-R_{680})/(R_{800}+R_{680})$ | NDVI | $y = -13695x^2 + 23241x - 9675.9$ | 0.3430a ns | 76.83 |
| $(R_{750}-R_{675})/(R_{750}+R_{675})$ | NDVI | $y = -35550x^2 + 56882x - 22613$ | 0.2989a ns | 78.73 |
| $(R_{750}-R_{680})/(R_{750}+R_{690})$ | NDVI | $y = -35550x^2 + 56882x - 22613$ | 0.2989a ns | 78.73 |

[z]Indices used in index development. $R_x$ = reflectance at wavelength x (in nm)

[y]VI (vegetation index or simple ratio), pigment specific simple ratio for Chl a (PSSR a) or Chl b (PSSR b), normalized difference vegetation index (NDVI), Chl Normalized difference index (Chl NDI), photochemical reflectance index (PRI), and structure insensitive pigment index (SIPI).

[x]Calibration equation for converting algorithm result to Chl a+b (y)

[w]Coefficient of determination ($R^2$) and Root mean square error (RMSE) values from regression of quadratic polynomial between leaf Chl a+b concentration and reflectance value transformation with different proposed indices, ns – not significant ($p>0.05$), **$p<0.01$, ***$p<0.001$; $R^2$ followed by the same letter are not significantly different ($p<0.05$, Fisher's Z-Test, n=72)

Table 4.6 The accuracy and calibration equations of the published indices after the Chl-related wavelength replaced by the optimal wavelength for assessing chlorophyll (Chl a, Chl b and Chl a+b) in poplar leaves

| Chl type | $OW_{Chl}$[z] | Indices[y] | Calibration equation[x] | $R^{2w}$ | RSME[w] ($\mu mol.m^{-2}$) |
|---|---|---|---|---|---|
| Chl a | 715nm | $R_{715}$ | $y = 851.93x^2 - 1296.8x + 462.95$ | 0.9604c*** | 24.51 |
| | | $R_{750}/R_{715}$ | $y = -11.525x^2 + 225.42x - 204.41$ | 0.9672c*** | 23.67 |
| | | $R_{800}/R_{715}$ | $y = -14.891x^2 + 225.55x - 200.13$ | 0.9674c*** | 23.65 |
| | | $R_{850}/R_{715}$ | $y = -13.423x^2 + 219.92x - 195.07$ | 0.9673c*** | 23.66 |
| | | $R_{940}/R_{715}$ | $y = -12.437x^2 + 216.13x - 189.45$ | 0.9685c*** | 23.51 |
| | | $R_{715}/R_{760}$ | $y = 483.84x2 - 1063.1x + 599.05$ | 0.9670bc*** | 23.70 |
| | | $R_{715}/R_{420}$ | $y = 1.9583x2 - 56.019x + 411.9$ | 0.9497c*** | 25.82 |
| | | $(R_{800}-R_{715})/(R_{800}+R_{715})$ | $y = 523.80x^2 + 358.10x + 12.244$ | 0.9673c*** | 23.66 |
| | | $(R_{750}-R_{715})/(R_{750}+R_{715})$ | $y = 588.06x^2 + 363.62x + 11.653$ | 0.9671c*** | 23.68 |
| | | $(R_{850}-R_{715})/(R_{850}+R_{715})$ | $y = 532.12x2 + 351.12x + 13.225$ | 0.9672c*** | 23.67 |
| Chl b | 730 nm | $R_{730}$ | $y = 2016.9x^2 - 1700.3x + 370.49$ | 0.8609a*** | 12.23 |
| | | $R_{750}/R_{730}$ | $y = 135.58x^2 - 341.74x + 227.7$ | 0.8666a*** | 12.10 |
| | | $R_{800}/R_{730}$ | $y = 120.13x^2 - 303.49x + 204.06$ | 0.8653a*** | 12.13 |
| | | $R_{850}/R_{730}$ | $y = 118.69x^2 - 299.59x + 201.43$ | 0.8645a*** | 12.15 |
| | | $R_{940}/R_{730}$ | $y = 117.24x^2 - 292.53x + 194.84$ | 0.8678a*** | 12.07 |
| | | $R_{730}/R_{760}$ | $y = 3512.4x2 - 6572.2x + 3086.7$ | 0.8657a*** | 12.12 |
| | | $R_{730}/R_{420}$ | $y = 5.9429x2 - 129.84x + 721.59$ | 0.8034a*** | 13.48 |
| | | $(R_{750}-R_{730})/(R_{750}+R_{730})$ | $y = 1438.5x^2 - 355.62x + 34.088$ | 0.8610a*** | 12.23 |
| | | $(R_{800}-R_{730})/(R_{800}+R_{730})$ | $y = 1326.6x^2 - 334.83x + 33.259$ | 0.8600a*** | 12.25 |
| | | $(R_{850}-R_{730})/(R_{850}+R_{730})$ | $y = 9607.6x2 - 605.83x + 21.928$ | 0.8572a*** | 12.31 |

| | | | | | |
|---|---|---|---|---|---|
| Chl a+b | 720nm | $R_{720}$ | $y = 2868.8x^2 - 2997.1x + 833.44$ | 0.9484bc*** | 33.22 |
| | | $R_{750}/R_{720}$ | $y = 124.06x^2 - 116.32x + 23.285$ | 0.9547bc*** | 32.60 |
| | | $R_{800}/R_{720}$ | $y = 2026.5x^2 + 8.0036x + 45.741$ | 0.9540bc*** | 32.67 |
| | | $R_{850}/R_{720}$ | $y = 105.27x^2 - 79.674x + 6.3681$ | 0.9542bc*** | 32.65 |
| | | $R_{940}/R_{720}$ | $y = 105.24x^2 - 77.936x + 3.9312$ | 0.9544bc*** | 32.63 |
| | | $R_{720}/R_{760}$ | $y = 1541.9x2 - 3009.4x + 1519.4$ | 0.9532bc*** | 32.75 |
| | | $R_{720}/R_{420}$ | $y = 5.4584x2 - 131.1x + 839.39$ | 0.9280b*** | 35.21 |
| | | $(R_{750}-R_{720})/(R_{750}+R_{720})$ | $y = 104.80x^2 - 76.40x + 5.3944$ | 0.9559bc*** | 32.48 |
| | | $(R_{800}-R_{720})/(R_{800}+R_{720})$ | $y = 1850.4x^2 + 23.272x + 45.503$ | 0.9537bc*** | 32.70 |
| | | $(R_{850}-R_{720})/(R_{850}+R_{720})$ | $y = 2896.3x2 + 194.98x + 41.413$ | 0.9531bc*** | 32.76 |

[z]$OW_{Chl}$ = 715, 730, and 720 for Chl a, Chl b, and Chl a+b, respectively.

[y]Algorithms used in index development. $R_x$ = reflectance at wavelength x (in nm)

[x]Calibration equation for converting algorithm result to Chl a, Chl b or Chl a+b (y)

[w]Coefficient of determination ($R^2$) and Root mean square error (RMSE) values from regression of quadratic polynomial between leaf Chl (Chl a, Chl b and Chl a+b) concentrations and reflectance value transformation with different proposed indices, ***$p<0.001$; $R^2$ followed by the same letter are not significantly different ($p<0.05$, Fisher's Z-Test, n=72)

Table 4.7 Coefficients of determination for relationships between concentrations of chlorophyll (Chl a, Chl b, and Chl a+b) and reflectance values for indices developed with optimal wavelength for chlorophyll assessment of ($OW_{Chl}$) in visible and red edge regions

| Chl type | $OW_{Chl}$ in visible region[z] | | $OW_{Chl}$ in NIR region[y] | |
|---|---|---|---|---|
| | Indices[x] | $R^{2w}$ | Indices[x] | $R^{2w}$ |
| Chl a | $R_{580}$ | 0.9497b*** | $R_{715}$ | 0.9604bc*** |
| Chl b | $R_{563}$ | 0.7440a** | $R_{730}$ | 0.7801a** |
| Chl a+b | $R_{574}$ | 0.9166b*** | $R_{720}$ | 0.9484bc*** |
| Chl a | $R_{368-466}/R_{580}$ | 0.9498-0.9610b*** | $R_{750-1100}/R_{715}$ | 0.9644-0.9697c*** |
| | $R_{750-1100}/R_{580}$ | 0.9621-0.9655b*** | | |
| Chl b | $R_{380-681}/R_{563}$ | 0.7458-0.8335a** | $R_{750-1100}/R_{730}$ | 0.7400-0.7819a** |
| | $R_{750-1100}/R_{563}$ | 0.7788-0.7907a** | $R_{409-448}/R_{730}$ | 0.7412-0.7854a** |
| Chl a+b | $R_{386-489}/R_{574}$ | 0.9306-0.9460b*** | $R_{423-425}/R_{720}$ | 0.9308-0.9316b*** |
| | $R_{750-1100}/R_{574}$ | 0.9370-0.9421b*** | $R_{750-1100}/R_{720}$ | 0.9425-0.9535bc*** |
| Chl a | $(R_{386-489}-R_{580})/(R_{386-489}-R_{580})$ | 0.9499-0.9619b*** | $(R_{750-1100}-R_{715})/(R_{750-1100}-R_{715})$ | 0.9644-0.9680c*** |
| | $(R_{750-1100}-R_{580})/(R_{750-1100}-R_{580})$ | 0.9499-0.9526b*** | | |
| Chl b | $(R_{380-679}-R_{563})/(R_{380-679}+R_{563})$ | 0.7462-0.8176a** | $(R_{409-448}-R_{730})/(R_{409-448}+R_{730})$ | 0.7401-0.7707a** |
| | | | $(R_{750-1100}-R_{730})/(R_{750-1100}+R_{730})$ | 0.7436-0.7824a** |
| Chl a+b | $(R_{386-489}-R_{574})/(R_{386-489}+R_{574})$ | 0.9300-0.9426b*** | $(R_{423-425}-R_{720})/(R_{423-425}+R_{720})$ | 0.9301-0.9308b*** |

[z]$OW_{Chl}$ in visible region is the optimal wavelength selected in the spectral visible region with largest and smallest RMSE for Chl assessment.
[y]$OW_{Chl}$ in NIR region is the optimal wavelength selected in the spectral NIR region with largest and smallest RMSE for Chl assessment.
[x]Indices used in for data transformation. $R_x$ = reflectance at wavelength x (in nm)
[w]Coefficient of determination ($R^2$) and Root mean square error (RMSE) values from simple linear regression between leaf Chl (Chl a, Chl b and Chl a+b) concentrations and reflectance value transformation with different indices. **p<0.01, ***p<0.001; $R^2$ followed by the same letter within same column are not significantly different (p<0.05, Fisher's Z-Test, n=72)

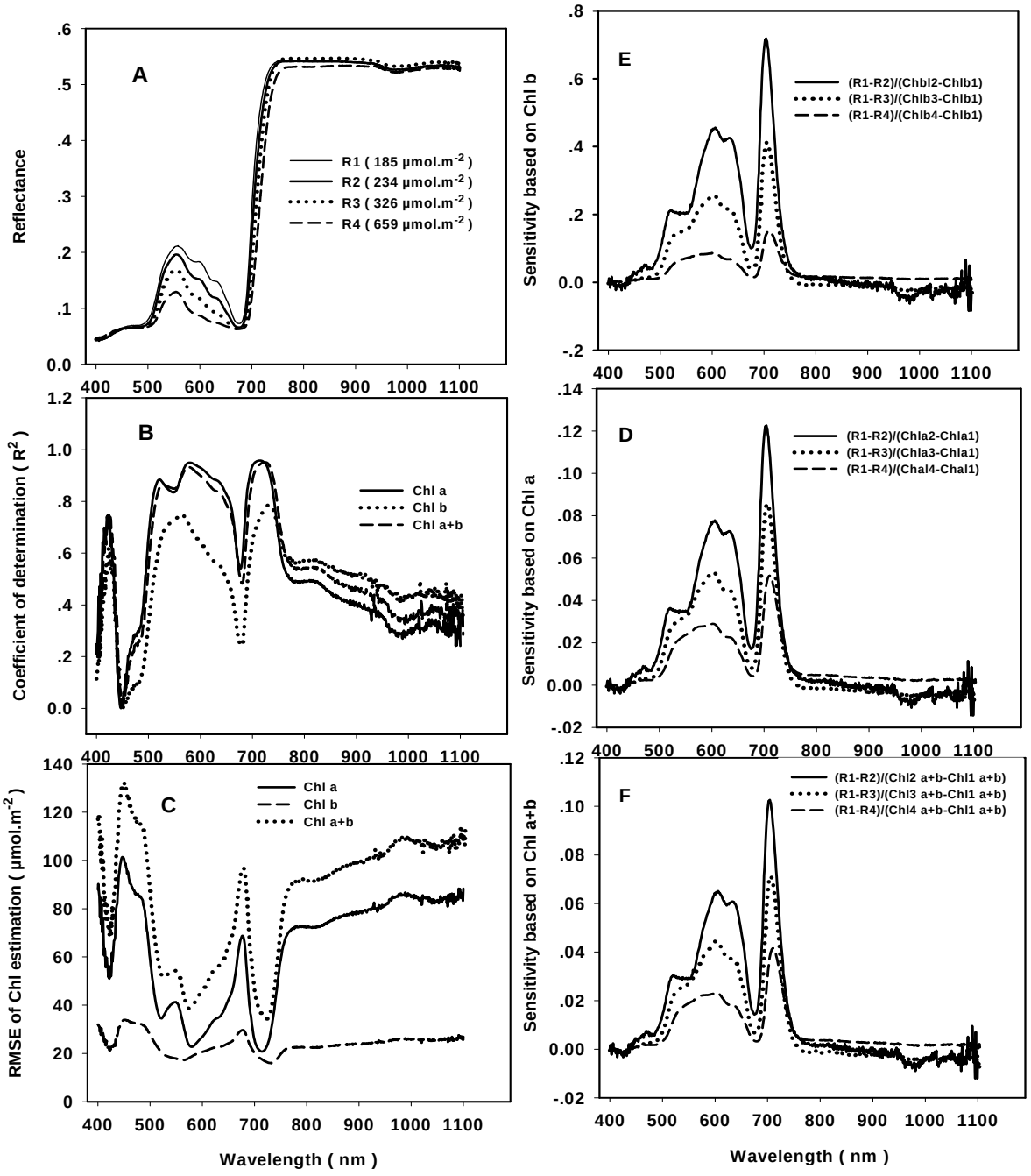


Figure 4.1 The (A) original reflectance spectrum from poplar leaves with different chlorophyll concentrations (Chl1-Chl4), the (B) coefficients of determination ($R^2$) and (C) root mean square error (RMSE) for relationships between reflectance values and concentrations of different Chl types (Chl a, Chlb, and Chl a+b) of 72 leaf samples, and the wavelength sensitivity of (D) Chl a, (E) Chl b and (F) Chl a+b at 1 nm intervals from 300 to 1100nm in poplar leaves. Sensitivity for each specific Chl type computed by dividing the reflectance difference (R1-R2, R1-R3, R1-R4) between leaf samples S2, S3, S4 and S1 for different Chl (Chl a, Chl b or Chl a+b) concentrations, then multiplying by 100.

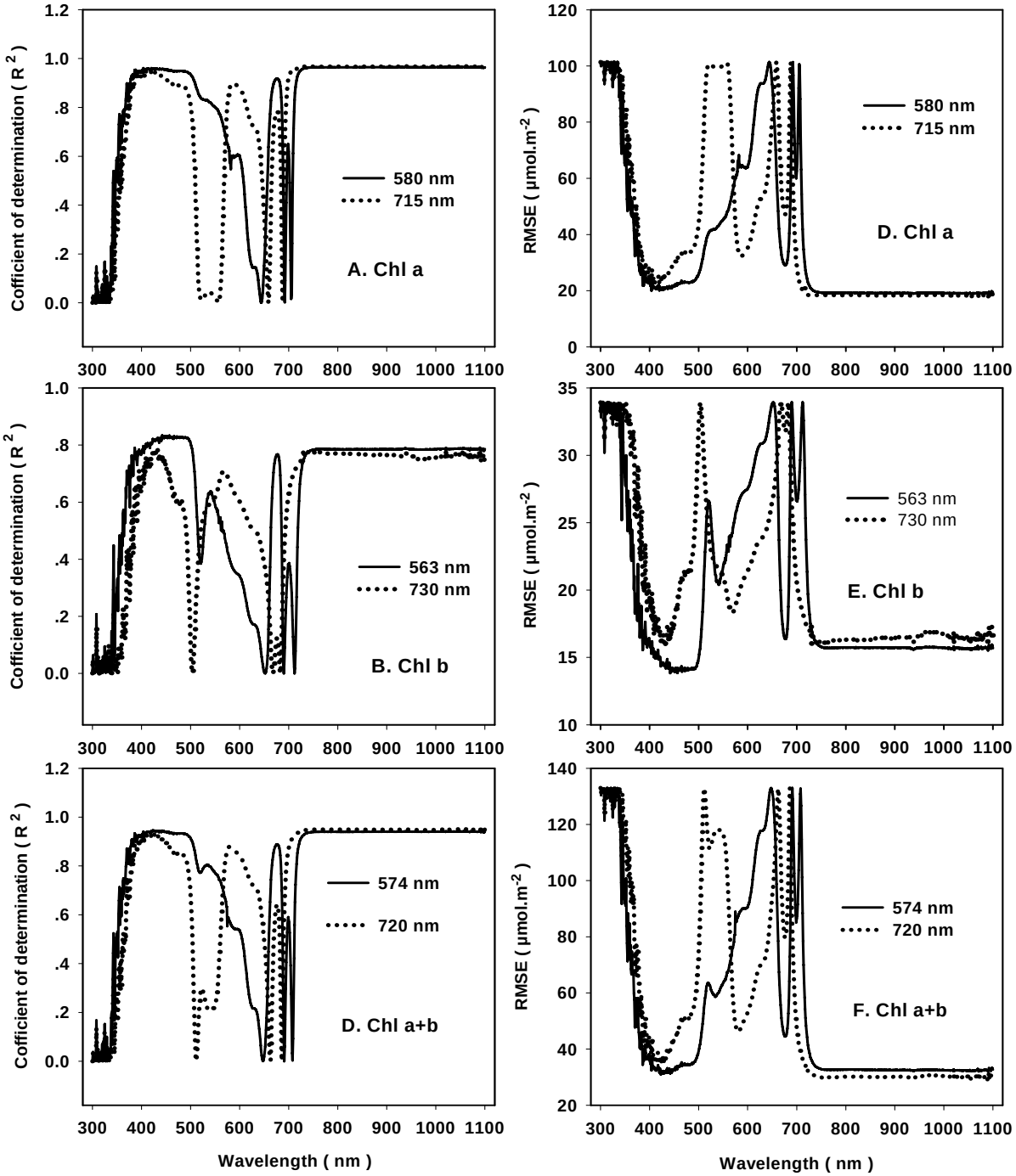


Figure 4.2 The coefficients of determination ($R^2$) (A, B, and C) and root mean square errors (RMSE) (C, D and E) from simple linear regressions between transformed reflectance values based on simple ratio and concentrations of chlorophyll (Ch a, Ch b and Ch a+b) in poplar leaves from 300 nm to 1100 nm with 1 nm intervals and sample number n=72. Reflectance of Chl a transformed using indices $R_{300\text{-}1100}/R_{580}$ and $R_{300\text{-}1100}/R_{715}$ with optimum wavelengths ($OW_{chl}$) 580 nm and 715 nm; reflectance of Chl b transformed using indices $R_{300\text{-}1100}/R_{563}$ $R_{300\text{-}1100}/R_{730}$ with $OW_{chl}$ 563 nm and 730 nm; reflectance of Chl a + b transformed using indices $R_{300\text{-}1100}/R_{574}$ and $R_{300\text{-}1100}/R_{720}$ with $OW_{chl}$ 574 nm and 720 nm, respectively.

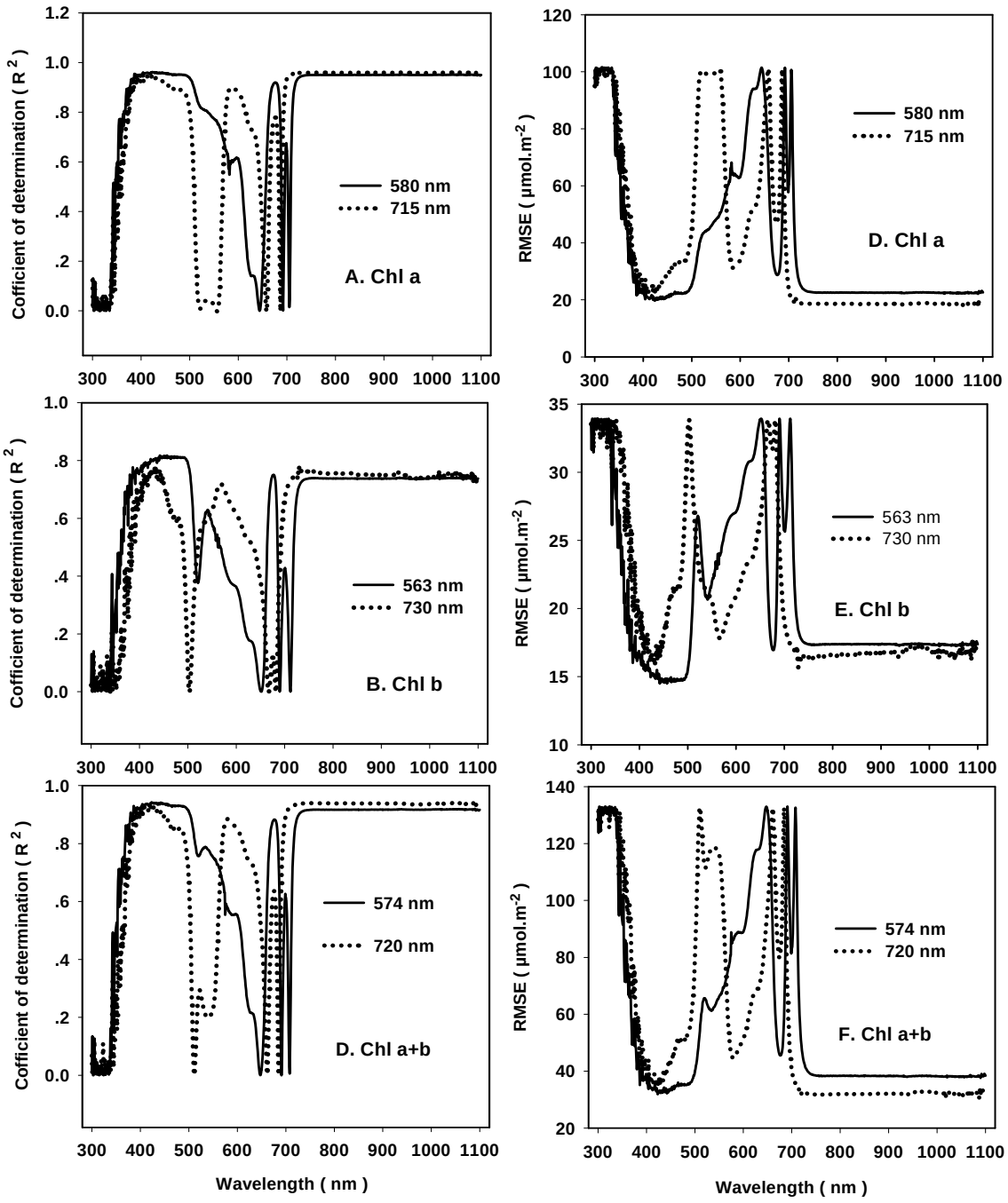


Figure 4.3 The coefficients of determination ($R^2$) (A, B, and C) and root mean square errors (RMSE) (C, D and E) from simple linear regressions between transformed reflectance values based on normalized difference vegetation index (NDVI) and concentrations of chlorophyll (Ch a, Ch b and Ch a+b) in poplar leaves from 300 nm to 1100 nm with 1 nm intervals and sample number n=72. Reflectance of Chl a transformed using indices $(R_{300\text{-}1100}\text{-}R_{580})/(R_{300\text{-}1100}+R_{580})$ and $(R_{300\text{-}1100}\text{-}R_{715})/(R_{300\text{-}1100}+R_{715})$ with optimum wavelengths ($OW_{chl}$) 580 nm and 715 nm; reflectance of Chl b transformed using indices $(R_{300\text{-}1100}\text{-}R_{563})/(R_{300\text{-}1100}+R_{563})$ and $(R_{300\text{-}1100}\text{-}R_{730})/(R_{300\text{-}1100}+R_{730})$ with $OW_{chl}$ 563 nm and 730 nm; reflectance of Chl a + b transformed using indices $(R_{300\text{-}1100}\text{-}R_{574})/(R_{300\text{-}1100}+R_{574})$ and $(R_{300\text{-}1100}\text{-}R_{720})/(R_{300\text{-}1100}+R_{720})$ with $OW_{chl}$ 574 nm and 720 nm, repectively.

# CHAPTER 5

# EFFECT OF LEAF PROPERTIES ON NONDESTRUCTIVE ASSESSMENT OF CHLOROPHYLL IN FRESH LEAVES USING SPECTRAL REFLECTANCE

## 5.1 Abstract

One-year-old 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, 15-year-old purple leaf plum (*Prunus cerasifera* 'Newport'), 10-year-old purple leaf flowering cherry (*Prunus blireiana*) grown in the campus landscape of Oregon State University and 8-years-old three apple cultivars (*Malus domestica* Borkh 'Jonagold','Gala' and 'Cameo') grown in orchard conditions were used to assess how (1) optimum wavelengths for chlorophyll assessment ($OW_{Chl}$) are influenced by plant genotype and (2) leaf properties (leaf texture, pigmentation, and water status) influence chlorophyll (Chl) assessment by altering reflectance characteristics of leaves. We determined that the accuracy of using optimum wavelengths for Chl assessment ($OW_{Chl}$) in both visible (540 - 580 nm) and red edge (700- 730 nm) regions varied among species and with Chl type (e.g. Chl a, Chl b, total Chl). Differences in $OW_{Chl}$ among species caused by the variation in leaf optical properties impaired the accuracy of indices used for assessing Chl. However, the $OW_{Chl}$ in the red edge region could be used accurately to measure Chl across all species tested; whereas the $OW_{Chl}$ in the visible region could be only used across a wide range of anthocyanins-free species. We determined that variation in reflectance of visible and red edge wavelengths caused by variation of leaf texture or other optical properties could be eliminated by referencing

the Chl-sensitive $OW_{Chl}$ to a NIR wavelength (750 – 1100 nm) that was sensitive to leaf texture but insensitive to Chl by using the following algorithms: $R_{540-580}/R_{750-1100}$, $R_{700-750}/R_{750-1100}$, $(R_{540-580}-R_{750-1100})/(R_{540-580}+R_{750-1100})$, or $(R_{700-740}-R_{750-1100})/(R_{700-740}+R_{750-1100})$. We also determined that the effect of dehydration on spectral reflectance can possibly be eliminated by referencing a Chl-sensitive $OW_{Chl}$ to a water sensitive wavelength between 1420 nm and 1510 nm. The effect of anthocyanins on the accuracy of Chl assessment differed among species and indices. Indices developed using $OW_{Chl}$ from the red edge were more accurate for assessing Chl and were less affected by the existence of anthocyanins, variation of leaf water status and other interferences than indices developed using $OW_{Chl}$ from the visible region. Carotenoid concentrations were positively correlated to Chl concentrations and did not influence the accuracy of the indices developed for Chl assessment using $OW_{Chl}$ from either the visible or red edge regions.

## 5.2 Introduction

Chlorophyll (Chl), the dominant and essential pigments of green leaves, determines, to a great extent, the amount of photosynethetically active radiation (PAR) absorbed by leaves, leaf photosynthetic rate, and plant productivity (Gamon and Qiu 1999, Kochubei 1990, Nichiporovich 1974, and Richardson et al. 2002). As the indicator of plant N status (Filella et al. 1995, Moran et al. 2000) and various stresses (Carter and Knapp 2001, Hendry 1987, Peñuelas and Filella 1998), Chl concentration in leaves changes throughout plant growth and development, during adaptation to unfavorable environmental conditions, and as a result of various stresses and damage (Demmig-Adams et al. 1996, Gamon and

Qiu 1999, Lichtenthaler 1996, Markstädter et al. 2001, Merzlyak et al. 1999, Penuelas and Filella 1998). Traditionally, Chl analysis is performed with spectrophotometry of Chl extracts in organic solvents (Arnon1949, Merzlyak et al. 2003). The application of this methodology involves tissue destruction, and is time-consuming and coupled with artifacts due to pigment instability, incomplete extraction, and the presence of light absorbing impurities (Lichtenthaler 1987, Solovchenko et al. 2001).

Recently, alternative nondestructive optical methods for Chl assessment, based on the absorbance and/ or reflectance of light by intact leaves, 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 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). Prior research has focused on the development and evaluation of Chl-related indices (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, these indices have rarely been tested using data from species other than those used in the formulation of the indices (Richardson et al 2002). There are many reasons why the proposed indices or algorithms are not applicable for Chl assessment across different studies and among different species. The main reason is that the optimum wavelengths for measuring Chl ($OW_{Chl}$) used in one study differ from those used in other studies. This is because $OW_{Chl}$ vary with the plant genotype and phenotype. Essentially, poor applicability of indices across genotypes and studies is a function of variation in leaf optical properties (leaf thickness, texture, density, Chl content, water

status, etc.). The objectives of this study were to (1) quantify how plant genotype influences $OW_{Chl}$ and the accuracy of Chl assessment; (2) determine the influence of leaf texture and leaf water status on Chl assessment; and (3) identify whether other pigments in leaves (e.g. carotenoids, anthocyanins) influence $OW_{Chl}$ and the accuracy of Chl assessment.

## 5.3 Materials and methods

### *5.3.1 Plant materials*

One-year-old 'Nonpareil' almond (*Prunus dulcis* (Mill.) D.A. Webb), poplar (UCC-1, a hybrid of *Populus trichocarpa* × *P. deltoides*, Union Camp, Princeton, NJ) and bench-grafted 'Fuji' apple (*Malus domestica* Borkh 'Fuji') trees 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-10$P_2O_5$-20$K_2O$ water-soluble fertilizer with micronutrients (Plantex Corp.,Ontario, Canada). When the new shoots were approximately 15 cm long, plants were moved to full sunlight and fertilized weekly with Plantex for 3 weeks. Beginning June 30, 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. At different dates in 1999, 2000, and 2002, 12 fresh leaves from each N fertigation treatment were removed from trees.

Leaves were also collected from 15 year old purple leaf plum (*Prunus cerasifera* 'Newport') and 10-year-old purple leaf flowering cherry (*Prunus blireiana*) growing in the campus landscape of Oregon State University under natural conditions and from 8-year-old three apple cultivars (*Malus domestica* Borkh 'Jonagold', *Malus domestica* Borkh 'Gala' and *Malus domestica* Borkh 'Cameo') growing in the Lewis Brown Horticultural Farm in Corvallis, Oregon (44° 33' N, 123° 46' W). After removal from trees, all leaves (72 per genotype) were immediately placed into plastic bags and transported to the laboratory for spectral reflectance, leaf Chl and other pigments analyses.

### *5.3.2 Spectral reflectance and leaf pigment analyses*

Discs were excised from leaves with a cork borer (2.85cm$^2$) and spectral reflectance of leaf discs was determined from 300 nm to 1100 nm at 1 nm intervals using Li-1800 spectroradiometer (Li-Cor Inc., Lincoln, NE). The reflectance spectrum for each leaf was calculated as $R_\lambda$= (leaf radiance at wavelength λ)/(reflectance standard radiance at wavelength λ), and was averaged across the two separate scans made on each leaf disc. The spectral reflectance of the same leaf discs was then determined from 400 nm to 2500 nm at 2 nm intervals using a FOSS NIRSystems 6500 scanning monochromator (FOSS NIRSystems, Laurel, MD). After scanning, each leaf disc was cut into small 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), Chl a, Chl b and total carotenoids concentrations were calculated according to Lichtenthaler and Wellburn

(1983), and anthocyanins were determined according to Abdel-Aal and Hucl (1999). Total Chl were calculated as the sum of Chl a and Chl b.

### *5.3.4 Effect of leaf texture on reflectance*

The NIR reflectance spectra (determined by the FOSS NIRSystems 6500 scanning monochromator) of leaves with similar Chl concentrations but differing reflectance (determined by spectroradiometry) were compared to assess the influence of leaf texture on reflectance.

### *5.3.5 Effect of leaf water status on leaf reflectance*

Six leaves with similar Chl contents from each genotype of 'Fuji' apple and purple leaf flowering cherry were used to assess the effect of leaf water status on spectral reflectance, respectively. Two leaf disks were excised from each leaf with a cork borer ($2.85cm^2$) from opposite sides of the main central vein. Fresh weights of discs were determined and the total Chl concentration of one disc from each leaf was determined using the methods outlined above. The other leaf disc was scanned from 400 nm to 2500 nm at 2 nm intervals using FOSS NIRSystems 6500 scanning monochromator. The scanned leaf discs were kept at 20 °C for 1 h then reweighed and rescanned to determine the spectral reflectance after dehydration. The leaves were then oven dried at 85 $^{o}$C for 48 h. Change in the weight of the leaf discs was used to assess the influence of leaf water status on reflectance. Leaf disc water status was expressed as percentage of water on a fresh weight basis. The mean reflectance results of six leaf discs for each genotype before and after dehydration at different wavelength was to calculate the effect of dehydration

on spectral reflectance, and the mean water content of six leaf discs was used to represent leaf water content of the genotype.

### *5.3.6 Regression analysis of spectral reflectance and Chl data*

Using Microsoft Visual Basic 6.0 (Microsoft Corp., Redmond, WA), custom software was developed to directly perform simple linear regression equations (linear-least-squares-fit) and calculate root mean square error (RMSE) and coefficients of determination ($R^2$) between the spectral reflectance reading from 300 nm to 1100 nm at 1 nm wavelength intervals 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. Pearson correlations among regressions were examined with the significance levels of $p<0.05$, $p<0.01$ and $p<0.001$. Difference between $R^2$ was tested as pairwise comparisons using Fisher's Z-test (Lawley 1938 ).

## 5.4 Results

### *5.4.1 Influence of plant genotype on $OW_{Chl}$ and accuracy of Chl assessment*

The $OW_{Chl}$ and $R^2$ for estimating Chl in visible and red edge regions varied among species and with Chl type (e.g. Chl a, Chl b, total Chl) (Table 5.1, Table 5.2). $R^2$–curves for anthocyanin-free species (apple, poplar and almond) showed two Chl-related peaks: one in the visible region (green to orange, 540 -581 nm) and one in the red edge (702 - 730 nm) of the spectra. $R^2$–curves for anthocyanin-rich species (purple leaf plum and purple leaf flowering cherry) showed a similar range of peak-related wavelengths in the red edge (712 -716 nm) and a different range of peak-related wavelength in visible region (580 - 636 nm) with longer wavelength than those of anthocyanin-free species. Within the same genotype the $R^2$ of $OW_{Chl}$ for estimating different Chl types were similar except the $R^2$ of 'Fuji' apple in the red edge, 'Jonagold' apple in the visible, and poplar in both visible and red edge regions. Within the same genotype, the $R^2$ of $OW_{Chl}$ in the red edge for estimating same Chl type was similar to those in the visible region for leaves of all genotypes except 'Fuji' apple and purple leaf plum, indicating the $OW_{Chl}$.in both regions were validated for assessing Chl in most species tested. The $R^2$ and RMSE of $OW_{Chl}$ for estimating different Chl types also differed among apple cultivars ('Fuji', 'Jonagold', 'Gala' and 'Cameo'), however the range of $OW_{Chl}$ among different cultivars was smaller in both the visible (540 -560nm) and red edge (702 - 720 nm) regions than the range amongst all species examined (540 -636 nm and 702 - 730 nm).

### *5.4.2 Influence of leaf texture on $OW_{Chl}$ and accuracy of Chl assessment*

The spectra determined using the FOSS NIR Systems 6500 scanning monochromator on leaves with different Chl concentrations showed that leaves with higher reflectance in the visible (540-600 nm) and red edge (700-740 nm) regions (as determined using

spectroradiometer) also had higher reflectance in the NIR region (750-1750 nm) of the spectrum (Figure 5.1).

Using the simple ratio ($R_{540-580}/R_{750-1100}$; $R_{700-740}/R_{750-1100}$) or normalized reflectance difference [$(R_{540-580}-R_{750-1100})/(R_{540-580}+R_{750-1100})$; $(R_{700-740}-R_{750-1100})/(R_{700-740}+R_{750-1100})$] indices decreased the effect of genotypic differences on Chl assessment and improved accuracy (larger $R^2$ and smaller RMSE) of Chl assessment for some species, especially when the $R^2$ is smaller (Table 5.3). For example, using the normalized reflectance difference index instead of just the $OW_{Chl}$ improved the accuracy of Chl assessment in almond using both visible and red edge wavelengths.

### *5.4.3 Influence of leaf water status on reflectance*

Dehydration increased leaf spectral reflectance of wavelengths in the visible (540 – 600nm), red edge (700 – 740 nm) and NIR (1350 – 2500 nm) regions for leaves of 'Fuji' apple and purple leaf flowering cherry (Figure 5.2). Spectral reflectance of 750 nm to 1000 nm wavelengths in the NIR region were not influence by 1 h dehydration at 20 °C.

### *5.4.4 Influence of Chl concentration and Chl a/b ratio on $OW_{Chl}$ and accuracy of Chl assessment*

Our results showed that red wavelengths 650 - 680 nm had smaller $R^2$ and larger RMSE than the $OW_{chl}$ we selected in the visible of 540-600 nm and the red edge of 700-730 nm regions (Figure 3.1 in Chapter 3, Table 5.1), indicating that the red wavelengths were not ideal wavelength for Chl assessment. This has been further verified by scatter plots comparison with the wavelength from red (e.g. 675 nm), and the optimum

wavelengths we selected from both red edge (e.g. 720 nm) and visible (e.g. 550 nm) (Figure 5.3). The wavelength in the visible and red edge regions had larger $R^2$ and smaller RMSE and $OW_{Chl}$ from these regions were accurate for assessing Chl across a wide range of Chl concentrations (160 to 1188μmol.$m^{-2}$) (Table 5.1, Table 5.4).

The $OW_{Chl}$ for Chl a in the visible region was 2 - 18 nm greater than the $OW_{Chl}$ for Chl b in leaves of 'Fuji' apple, poplar and purple leaf flowering cherry; whereas $OW_{Chl}$ for Chl a was 6-9 nm smaller than that of Chl b in leaves of almond and purple leaf plum (Table 5.4). The variation of $OW_{Chl}$ in the red edge region was smaller among species than that in the visible region; and the $OW_{Ch}$-related $R^2$ of Chl a and Chl a+b in the red edge for leaves of apple, poplar and purple leaf flowering cherry were also greater than those for leaves of almond and purple leaf plum.

Among anthocyanin-free species (e.g. poplar, apple, almond), leaves with higher Chl a/b ratios had a longer $OW_{Chl}$ in the visible region (Table 5.4). However, leaves of purple leaf plum and purple leaf flowering cherry had much smaller Chl a/b ratios than the anthocyanin-free species and their $OW_{Chl}$ were shifted more from the in the visible green region toward the longer wavelength of red region of the spectrum in comparison to the $OW_{Chl}$ of anthocyanin-free species. For all species the $OW_{Chl}$ in the red edge region of the spectrum was not affected by Chl a/b ratio in leaves.

### *5.4.5 Influence of anthocyanins and carotenoids on $OW_{Chl}$ and accuracy of Chl assessment*

From July to August, almost no anthocyanins could be detected in the leaves of 'Fuji' apple, poplar and almond. However, the leaves of purple leaf plum and purple leaf

flowering cherry contained approximately 145.56 - 624.93μmol.m$^{-2}$ and 157.17 - 297.25μmol.m$^{-2}$ of anthocyanins, respectively (Table 5.4). The $R^2$-curves based on Chl concentrations showed two peaks in all the plant species tested; however the peaks in the $R^2$-curves for leaves of 'Fuji' apple, poplar and purple leaf flowering cherry were significantly larger than peaks for leaves from purple leaf plum (Table 5.4, Figure 5.4).

The $OW_{Chl}$ in the red edge (710–730 nm) for the leaves of anthocyanin-free species (e.g. 'Fuji' apple, poplar and almond) were only a few nanometers different from the $OW_{Chl}$ in the red edge (712-716 nm) for the leaves from the anthocyanin-rich species (e.g. purple leaf plum and purple leaf flowering cherry) (Table 5.4). The $OW_{Chl}$ in the visible region (580-636 nm) of the anthocyanin-rich species was at longer wavelengths than the $OW_{Chl}$ (550-581 nm) of the anthocyanin-free species (Table 5.4).

$OW_{Chl}$ from the red edge (700- 730 nm) can be used to accurately assess Chl across all species we tested; whereas $OW_{Chl}$ in the visible (540 – 580 nm) would be better when used with the anthocyanin-free species. The $R^2$-curves based on anthocyanin concentrations from leaves of anthocyanin-rich species showed two major peaks that overlapped with the $R^2$-curves based on Chl concentrations. In leaves of purple leaf flowering cherry the $OW_{Chl}$ in both visible and red edge regions had high $R^2$ values and were not affected by anthocyanin concentrations of 157.17 - 297.25μmol.m$^{-2}$ (Table 5.4 and Figure 5.4).

The anthocyanin concentrations in the leaves of purple leaf flowering cherry were positively correlated to Chl concentrations ($R^2$= 0.5512; $P<0.01$, n=72). In contrast, the anthocyanin concentrations in leaves of purple leaf plum showed no relationship to Chl concentrations ($R^2$=0.1933; $P>0.05$, n=72).

The $R^2$-curves based on carotenoid concentration from leaves showed two major peaks that overlapped on the wavelength with the $R^2$-curves based on Chl concentrations in both visible and red edge regions (Figure 5.4). Carotenoid concentrations in leaves of different genotypes ('Fuji' apple, poplar, purple leaf plum and purple leaf flowering cherry) were positively correlated to Chl concentrations ($R^2$= 0.7928-0.8763, $P<0.01$, n=72).

## 5.5 Discussion

We found that using $OW_{Chl}$ in indices was very important for developing accurate indices for Chl assessment across different species. Our results indicate that there are two $OW_{Chl}$ for each species: one in visible (550– 636 nm) and one in the red edge (702– 730 nm) region of the spectrum (Table 5.1 and Table 5.2). An important objective when developing indices to estimate Chl is to determine which indices can be used across a wide range of species and functional groups (Richardson et al. 2002). However, this objective has been proven difficult to achieve (Richardson et al. 2002). Gamon and Surfus (1999) demonstrated that the relationship between normalized difference vegetation index (NDVI) and total Chl is markedly different for a conifer, *Pseudotsuga menziesii* and a herbaceous plant, *Helianthus annuus*; they suggest this may be due to differences in leaf morphology and structure. We found that although the $OW_{Chl}$ for all the species tested were in these two narrow regions, differences in $OW_{Chl}$ among species was enough to influence the accuracy ($R^2$ and RMSE) of Chl assessment and impair the accuracy of an index or calibration equation when used across a wide range of vegetation types. Richardson et al. (2002) also found similar effects of the differences in leaf structure on

indices accuracy across a wide range of vegetation types in remote sensing. We found that variation in $OW_{Chl}$ also exist among cultivars within the same species (Table 5.1), although the variation among cultivars within the same species is much smaller than among species (Table 5.2). Therefore, in order to accurately assess Chl in leaves, selecting the Chl related $OW_{Chl}$ to develop an algorithm for a specific cultivar is important. However our results also indicate that the $OW_{Chl}$ from the red edge (700- 730 nm) can be used to accurately assess Chl across all species and Chl types; whereas $OW_{Chl}$ in the visible (540 – 580 nm) can be used to assess Chl in the anthocyanin-free species. These results validate $OW_{Chl}$.in both visible region and red edge regions are narrow enough for developing optimum indices for specific genotype or Chl type, and wide enough for developing "common" optimum indices to be consistent across genotypes and Chl types.

We found the $OW_{Chl}$ in the red edge for all species tested was narrower (702-730 nm) and more tolerate to influence factors (e.g. the existence of anthocyanins) than in the visible region (550-636 nm) (Table 5.1, Figure 3.1, Figure 5.4). Curran et al. (1990) reported the Chl a/b ratio increase resulted in the $OW_{Chl}$ shifting to longer wavelengths; conversely, Chl a/b ratio decrease resulted in the $OW_{Chl}$ shifting to shorter wavelengths. Our results indicate that the $OW_{Chl}$ in red edge region was not affected by Chl a/b ratio. The $OW_{Chl}$ in visible region was influenced by both the Ch a/b ratio and the existence of anthocyanins. Therefore, we conclude that indices developed with $OW_{Chl}$ in the red edge region (e.g. 710 nm) could be used to accurately assess leaf Chl of both anthocyanins-free and anthocyanin-rich species; whereas indices developed with $OW_{Chl}$ in the visible region (e.g. 565 nm) should only be used across anthocyanin-free species. This is very important

for indices development used for Chl assessment and for instrumentation. With the indices developed with the $OW_{Chl}$ in the red edge region, we could accurately assess Chl concentration in fresh leaves.

Leaf Chl concentration is another important factor affecting indices accuracy for Chl assessments. In earlier published investigations reflectance wavelengths from 670 - 680 nm were employed for Chl assessment (Merzlyak et al. 2003). Although the indices developed with these wavelengths showed a good sensitivity and linearity at low Chl concentrations, they became rapidly saturated and less accurate with an increase in Chl concentration over 100-150 $\mu g.m^{-2}$ (Gitelson et al. 1996, Gitelson and Merzlyak 1994, Buschmann and Nagel 1993). Our results showed that reflectance measurements using $OW_{Chl}$ in the visible (540-580 nm) and red edge (700-730 nm) regions were more accurate (e.g. higher $R^2$ and lower RSME) for Chl assessment than those from 670 - 680 nm and were sensitive across a wider range of Chl concentrations (160 to 1188 $\mu mol.m^{-2}$) (Table 5.1, Table 5.2, and Table 5.3). It should be noted that Chl absorption coefficients are very low at these wavelengths (Merzlyak et al. 2003). Contrarily, leaf spectra reflectance at these wavelengths are strong and we found reflectance decreased proportionally with increasing leaf Chl concentration and the reflectance response was not easily saturated by high Chl concentrations. Therefore, we conclude that indices developed with these wavebands can be used accurately to assess leaf Chl over a wider range of concentrations than wavelengths used for reflectance in other studies.

Leaf morphology and internal texture is another factor affecting leaf Chl reflectance assessment. One of the requirements of reliable algorithms for use in pigment analysis is their low sensitivity to morphological–anatomical traits of plant tissues (Merzlyak et al.

2003). Our results indicate that there is little variation in reflectance at NIR wavelengths (750 - 1100 nm). The leaves with similar Chl concentration and high reflectance in the visible (540-580nm) and red edge (700-730nm) regions also had high reflectance in NIR (750-1750nm) region (Figure 5.1). We believe the factors that govern the behavior of reflectance in the visible and red edge regions are different from the factors that govern the behavior of reflectance of 750 - 1750 nm wavelengths in the NIR. In this NIR region, an increase in reflectance might be caused by an increase in leaf thickness or density; in the visible and red edge regions, an increase in reflectance indicates a decrease in the concentration of Chl or other pigments. In leaves with the same Chl concentration, an increase in leaf thickness might cause an increase in reflectance in the Chl-insensitive NIR region and a relative decrease in Chl concentration on a unit volume basis. This would cause an increase in reflectance in the visible and red regions as shown in our results (Figure 5.1). With variation in leaf texture, reflectance in the visible, red edge, and NIR regions varied in the same manner; therefore, by referencing visible or red edge wavelengths that are sensitive to Chl to a NIR wavelength that is sensitive to leaf texture but insensitive to Chl, the effect of leaf texture on Chl assessment could be reduced.

Our results indicated that use indices of a simple ratios (e.g. $R_{540-580}/R_{750-1100}$, $R_{700-730}/R_{750-1100}$) or normalized reflectance difference [e.g. $(R_{540-580}-R_{750-1100})/(R_{540-580}+R_{750-1100})$, $(R_{700-730}-R_{750-1100})/(R_{700-730}+R_{750-1100})$] can improve the accuracy of Chl assessment (Table 5.3). It is noteworthy that we also found that the ratios $R_{540-580}/R_{750-1100}$ and, $R_{700-720}/R_{750-1100}$ possess similar sensitivity to Chl concentrations. The strength of these SR is the result of the strong positive correlation in healthy anthocyanin-free leaves between reflectance coefficients of 540 –580 nm and 700 - 730 nm wavelengths.These results

verified that the most commonly used indices used in remote sensing (SR and normalized reflectance difference) could be used to accurately measure leaf-level Chl concentration if developed with $OW_{Chl}$ either in visible (540 –580 nm) or red edge (700 - 730 nm) regions.

Leaf water status is another important factor affecting Chl assessment. We found dehydration reduced light penetration into leaves which in turn increased spectral reflectance in the visible (540-600nm), red edge (700-740nm) and NIR (1350-2500nm) regions in leaves of 'Fuji' apple and purple leaf flowering cherry (Figure 5.2). This is possibly a result of dehydration causing leaf cells to shrink and thereby increasing leaf density. However, leaf spectral reflectance at wavelengths sensitive to leaf texture (e.g. 750 nm - 1000 nm) was not influenced by dehydration. We determined that the most sensitive wavelengths to changes in leaf water status were from 1420 nm to 1510 nm. Therefore, the effect of leaf water status on reflectance could potentially be reduced by referencing the reflectance of a Chl sensitive wavelength from either visible or red edge region to a water-sensitive wavelength between 1420 nm and 1510 nm. The ability of using this water sensitive wavelength to improve indices accuracy has not been tested..

Plant leaves contain a number of pigments besides Chl. Anthocyanins and carotenoids are two important group pigments that absorb radiation and could therefore affect the accuracy of nondestructive assessment of leaf Chl. Anthocyanins (represented mainly by cyanidin derivatives) localized in vacuoles within leaf cells are reported to possess an absorption maximum near 540–550 nm (Gitelson et al. 2001, Merzlyak and Chivkunova 2000).We found that the anthocyanins in the species we tested possess a wider absorption range from 500 nm to 560 nm that overlapped with the $OW_{Chl}$ in the

visible region (Figure 5.4). Since absorption of Chl was also significant at these wavelengths, the contribution of anthocyanins to reflectance significantly decreases accuracy in Chl assessment of anthocyanins-rich leaves (Table 5.4). In the leaves of purple leaf plum, the accuracy of using $OW_{Chl}$ from either the visible or red edge region was decreased by the existence of anthocyanins (Figure 5.4, Table 5.4). Current et al (1990) also found the linear relationship between reflectance of wavelengths from the red edge and Chl concentrations was severely affected by the existence of anthocyanins in *Amaranthus tricolor* at low anthocyanin concentrations ($0.01 mg.g^{-1}$). However, we found that in leaves of purple leaf flowering cherry, the accuracy of using $OW_{Chl}$ from either the visible or red edge region for Chl assessment was not affected by the existence of anthocyanins at concentrations between $157.17 \sim 297.25 \mu mol.m^{-2}$.

The different effects of anthocyanins on the accuracy of Chl assessment between purple leaf flowering cherry and purple leaf plum may be related to differences in the relationship between Chl and anthocyanins in these species. Anthocyanin concentrations in leaves of purple leaf flowering cherry were positively correlated to Chl concentrations, while there was no relationship between Chl and anthocyanins in leaves of purple leaf plum. The different influence of anthocyanins on purple leaf flowering cherry and purple leaf plum may be caused by the difference in leaf structure and the distribution of pigments within the leaves.

The lack of relationship between Chl and anthocyanins in the leaves of purple leaf plum could decrease the accuracy of Chl assessment and the $OW_{Chl}$ selected for use in indices (Current et al. 1991). For example the accumulation of anthocyanin in purple leaf plum decreased the accuracy of using $R_{540-580} / R_{750-1100}$ for Chl assessment, but had little

influence on the accuracy of using $R_{700\text{-}730}$ / $R_{750\text{-}1100}$ (Figure 5.4, Table 5.4). Gitelson et al (2001) also reported that using the algorithm $R_{NIR}$ / $R$ 700 was better than $R_{NIR}$ / $R_{550}$ for assessing Chl concentrations in leaves that contained anthocyanin. In our experiment carotenoid concentrations were positively correlated to Chl concentrations and did not influence the accuracy of using $OW_{Chl}$ from either the visible or red region for Chl assessment.

## 5.6 Conclusions

Differences in $OW_{Chl}$ among and within species are enough to impair the accuracy of an index when used across a wide range of species. $OW_{Chl}$ from the red edge (700- 730 nm) region can be used to accurately assess Chl across all species we tested; whereas $OW_{Chl}$ in the visible (540 – 580 nm) region would be better used with the anthocyanin-free species. Referencing the $OW_{Chl}$ from either visible or red edge regions to specific wavelengths in the NIR can improve the accuracy of indices for Chl assessment by accounting for the influence of leaf texture (750 – 1100 nm) or dehydration (1420 - 1510 nm).

## 5.7 References


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Table 5.1 Correlation coefficients ($R^2$) and root mean square error (RMSE) of simple linear regression for the relationship between chlorophyll (Chl a, Chl b and Chl a+b) concentrations in leaves from five plant species and reflectance values at optimum wavelength ($OW_{Chl}$) in the visible and red edge regions of the spectrum for estimating different chlorophyll (Chl).

| Species[z] | Chl | Visible region (550 – 636 nm) | | | Red edge (710 – 730 nm) | | |
|---|---|---|---|---|---|---|---|
| | | $OW_{Chl}$[y] (nm) | $R^{2x}$ | RMSE[x] ($\mu mol.m^{-2}$) | $OW_{Chl}$[y] (nm) | $R^2$ | RMSE[x] ($\mu mol.m^{-2}$) |
| Almond | Chl a | 550 | 0.8740cd*** | 29.78 | 710 | 0.8678bcd*** | 29.80 |
| | Chl b | 558 | 0.8256bc*** | 18.77 | 710 | 0.8144bc*** | 19.03 |
| | Chl a + b | 550 | 0.8737cd*** | 47.55 | 710 | 0.8668bcd*** | 48.83 |
| Apple | Chl a | 552 | 0.7785b*** | 96.61 | 720 | 0.9198de*** | 57.69 |
| | Chl b | 550 | 0.7225b** | 26.94 | 717 | 0.8465bc*** | 19.59 |
| | Chl a + b | 552 | 0.7696b*** | 122.07 | 720 | 0.9073de*** | 75.76 |
| Poplar | Chl a | 581 | 0.9497e*** | 22.56 | 715 | 0.9579f*** | 18.97 |
| | Chl b | 563 | 0.7440b** | 15.67 | 730 | 0.7801b*** | 14.51 |
| | Chl a + b | 575 | 0.9166de*** | 38.23 | 720 | 0.9355def*** | 33.48 |
| Purple leaf plum | Chl a | 630 | 0.5392a* | 39.82 | 712 | 0.6027a** | 37.01 |
| | Chl b | 636 | 0.5105a* | 16.29 | 714 | 0.5870a** | 14.96 |
| | Chl a + b | 636 | 0.5354a* | 55.62 | 714 | 0.6020a** | 51.43 |
| Purple leaf flowering cherry | Chl a | 592 | 0.9388e*** | 26.64 | 716 | 0.9291df*** | 28.68 |
| | Chl b | 580 | 0.9207de*** | 11.14 | 714 | 0.8750cd*** | 13.99 |
| | Chl a + b | 590 | 0.9339e*** | 37.79 | 714 | 0.9170de*** | 42.37 |

[z]Poplar = *Populus trichocarpa* x *P. deltoids* 'UCC-1'; Apple = *Malus domestica* 'Fuji'; Almond = *Prunus dulcis* 'Nonpareil', Purple leaf flowering cherry **=** *Prunus blireiana* **;** Purple leaf plum = *Prunus cerasifera* 'Newport'

[y]$OW_{Chl}$ = optimum wavelength for Chl assessment determined by regression

[x]$R^2$ and RSME from regression of reflectance values on Chl concentration; * $p<0.05$, ** $p<0.01$, *** $p<0.001$; $R^2$ values followed by the same letter within a column not significantly different ($p<0.05$, Fisher's Z-Test, n=72)

Table 5.2 Correlation coefficients ($R^2$) and root mean square error (RMSE) of simple linear regression for the relationship between chlorophyll (Chl a, Chl b and Chl a+b) concentrations in leaves of four apple cultivars and reflectance values at optimum wavelength ($OW_{Chl}$) in the visible and red edge regions of the spectrum for estimating different chlorophyll (Chl).

| Cultivars[z] | Chl | Visible region (540 – 560 nm) | | | Red edge (702 – 720 nm) | | |
|---|---|---|---|---|---|---|---|
| | | $OW_{Chl}$[y] (nm) | $R^{2x}$ | RMSE[x] ($\mu mol.m^{-2}$) | $OW_{Chl}$[y] (nm) | $R^{2x}$ | RMSE[x] ($\mu mol.m^{-2}$) |
| Cameo | Chl a | 560 | 0.8804de*** | 92.32 | 716 | 0.9175bc*** | 62.57 |
| | Chl b | 550 | 0.8095abcd*** | 26.78 | 708 | 0.8612ab*** | 23.45 |
| | Chl a + b | 560 | 0.8643cde*** | 119.11 | 714 | 0.9023abc*** | 85.23 |
| Fuji | Chl a | 552 | 0.7785abc*** | 96.61 | 720 | 0.9198c*** | 57.69 |
| | Chl b | 550 | 0.7225a*** | 26.94 | 717 | 0.8465a*** | 19.59 |
| | Chl a + b | 552 | 0.7696ab*** | 122.07 | 720 | 0.9073abc*** | 75.76 |
| Gala | Chl a | 540 | 0.8492bcd*** | 80.12 | 708 | 0.8773abc*** | 72.26 |
| | Chl b | 552 | 0.8294abcd*** | 34.95 | 710 | 0.8637abc*** | 31.24 |
| | Chl a + b | 540 | 0.8453bcd*** | 115.05 | 708 | 0.8755abc*** | 103.5 |
| Jonagold | Chl a | 558 | 0.9527g*** | 55.59 | 710 | 0.9659d*** | 47.22 |
| | Chl b | 560 | 0.9168ef*** | 28.54 | 702 | 0.9139bd*** | 29.04 |
| | Chl a + b | 558 | 0.9505fg*** | 84.13 | 708 | 0.9581d*** | 76.26 |

[z] Cameo = *Malus domestica* 'Cameo' on M9 rootstock; Fuji = *Malus domestica* 'Fuji' on M9 rootstock; Gala = *Malus domestica* 'Gala' on M9 rootstock; Jonagold = *Malus domestica* 'Jonagold' on M9 rootstock

[y] $OW_{Chl}$ = optimum wavelength for Chl assessment determined by regression

[x] $R^2$ and RSME from regression of reflectance values on Chl concentration; ***$p<0.001$; $R^2$ values followed by the same letter within a column not significantly different ($p<0.05$, Fisher's Z-Test, n=72)

Table 5.3 Correlation coefficients ($R^2$) and root mean square error (RMSE) of simple linear regression for different indices used to estimate chlorophyll (Chl) in leaves of five species.

| Species[z] | Indices[w] | Visible region wavelengths | | | Red edge wavelengths | | |
|---|---|---|---|---|---|---|---|
| | | $OW_{Chl}$[y] (nm) | $R^2$[x] | RMSE[x] ($\mu mol.m^{-2}$) | $OW_{Chl}$[y] (nm) | $R^2$[x] | RMSE[x] ($\mu mol.m^{-2}$) |
| Almond | $R_{OW}$ | 550 | 0.8737cde$^{***}$ | 47.55 | 710 | 0.8667b$^{***}$ | 48.83 |
| | $R_{850}/R_{OW}$ | | 0.9346ef$^{***}$ | 34.19 | | 0.9393bcd$^{***}$ | 32.98 |
| | $(R_{850}-R_{OW})/(R_{850}+R_{OW})$ | | 0.9215f$^{***}$ | 37.48 | | 0.9278cd$^{***}$ | 35.95 |
| Apple | $R_{OW}$ | 552 | 0.7696ab$^{**}$ | 122.07 | 720 | 0.9072bc$^{***}$ | 75.76 |
| | $R_{850}/R_{OW}$ | | 0.8611bc$^{***}$ | 94.73 | | 0.9371cd$^{***}$ | 63.74 |
| | $(R_{850}-R_{OW})/(R_{850}+R_{OW})$ | | 0.8067bcd$^{***}$ | 111.80 | | 0.9370cd$^{***}$ | 63.81 |
| Poplar | $R_{OW}$ | 575 | 0.9166def$^{***}$ | 38.23 | 720 | 0.9352cd$^{***}$ | 33.48 |
| | $R_{850}/R_{OW}$ | | 0.9390def$^{***}$ | 32.60 | | 0.9480cd$^{***}$ | 30.09 |
| | $(R_{850}-R_{OW})/(R_{850}+R_{OW})$ | | 0.9157f$^{***}$ | 38.33 | | 0.9405d$^{***}$ | 32.21 |
| Purple leaf plum | $R_{OW}$ | 636 | 0.5354a$^{*}$ | 55.62 | 714 | 0.6027a$^{**}$ | 51.43 |
| | $R_{850}/R_{OW}$ | | 0.3612a$^{ns}$ | 65.22 | | 0.5168a$^{**}$ | 56.72 |
| | $(R_{850}-R_{OW})/(R_{850}+R_{OW})$ | | 0.3610a$^{ns}$ | 65.23 | | 0.5143a$^{**}$ | 56.87 |
| Purple leaf flowering cherry | $R_{OW}$ | 590 | 0.9339ef$^{***}$ | 37.79 | 714 | 0.9170cd$^{***}$ | 42.37 |
| | $R_{850}/R_{OW}$ | | 0.9431f$^{***}$ | 35.08 | | 0.9436cd$^{***}$ | 34.93 |
| | $(R_{850}-R_{OW})/(R_{850}+R_{OW})$ | | 0.9423f$^{***}$ | 35.32 | | 0.9393cd$^{***}$ | 36.23 |

[z]Poplar = *Populus trichocarpa* x *P. deltoids* 'UCC-1'; Apple = *Malus domestica* 'Fuji'; Almond = *Prunus dulcis* 'Nonpareil', Purple leaf flowering cherry = *Prunus blireiana*; Purple leaf plum = *Prunus cerasifera* 'Newport'

[y]$OW_{Chl}$ = optimum wavelength for Chl assessment determined by regression

[x]$R^2$ and RSME from regression of reflectance values on Chl concentration; * $p<0.05$, ** $p<0.01$, *** $p<0.001$; $R^2$ values followed by the same letter within a column not significantly different ($p<0.05$, Fisher's Z-Test, n=72)

[w]Indices used in regression analysis to calculate the $R^2$ and RMSE used for developing the $R^2$-curve vs wavelength from 400 to 2500 nm.

Table 5.4 Leaf pigment concentrations of chlorophyll a (Chl a); chlorophyll b (Chl b); total chlorophyll (Chl a+b); anthocyanins (Anth); carotenoids (Caro) and their and ratios in leaves of five species and the correlation coefficients ($R^2$) and root mean square error (RMSE) for the relationships between pigment concentrations and reflectance at the optimum wavelength ($OW_{Chl}$) for assessing chlorophyll (Chl).

| Species[z] | Pigments[y] | Concentration Range & ratio[x] ($\mu mol.m^{-2}$) | Mean[x] ($\mu mol.m^{-2}$) | Visible $OW_{Chl}$[w] (nm) | $R^{2v}$ | RMSE[v] ($\mu mol.m^{-2}$) | Red edge $OW_{Chl}$[w] (nm) | $R^{2v}$ | RMSE[v] ($\mu mol.m^{-2}$) |
|---|---|---|---|---|---|---|---|---|---|
| Almond | Chl a | 133.35~552.90 | 343.13 | 550 | 0.8740c*** | 36.76 | 710 | 0.8678bcde*** | 37.69 |
| | Chl b | 39.97~157.55 | 98.76 | 558 | 0.8256bc*** | 10.79 | 710 | 0.8144bc*** | 11.14 |
| | Total Chl | 173.32~710.45 | 441.89 | 550 | 0.8737c*** | 47.55 | 710 | 0.8667bcde*** | 48.83 |
| | Caro | 163.01~323.34 | 243.18 | | | | | | |
| | Total Chl/Caro | 1.06~2.20 | 1.63 | | | | | | |
| | Chla / Chlb | 3.34~3.51 | 3.42 | | | | | | |
| 'Fuji' apple | Chl a | 200.16~926.40 | 563.28 | 552 | 0.7785b*** | 96.61 | 720 | 0.9198ef*** | 57.69 |
| | Chl b | 61.39~262.33 | 161.86 | 550 | 0.7225b*** | 26.94 | 717 | 0.8465bcd*** | 19.59 |
| | Total Chl | 261.55~1188.72 | 725.14 | 552 | 0.7695b*** | 122.07 | 720 | 0.9072bdef*** | 75.76 |
| | Caro | 121.09~314.26 | 217.68 | | | | | | |
| | Chla / Chlb | 3.26~ 3.53 | 3.40 | | | | | | |
| | Total Chl /Caro | 2.16~3.78 | 2.97 | | | | | | |
| Poplar | Chl a | 131.22~509.58 | 320.40 | 581 | 0.9497d*** | 21.79 | 715 | 0.9579g*** | 18.97 |
| | Chl b | 24.64~149.95 | 87.30 | 563 | 0.7440b*** | 16.44 | 730 | 0.7801b*** | 14.51 |
| | Total Chl | 160.45~659.54 | 410.00 | 575 | 0.9166cd*** | 38.23 | 720 | 0.9352fg*** | 33.48 |
| | Caro | 80.22~153.80 | 117.01 | | | | | | |
| | Chla / Chlb | 5.33~3.40 | 4.36 | | | | | | |
| | Total Chl /Caro | 2.00~4.29 | 3.14 | | | | | | |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Purple leaf plum | Chl a | 120.94~366.81 | 248.92 | 630 | 0.5392a* | 39.82 | 712 | 0.6020a** | 37.01 |
| | Chl b | 44.24~138.69 | 99.33 | 636 | 0.5105a* | 16.29 | 714 | 0.5870a** | 14.96 |
| | Total Chl | 165.18~505.50 | 348.25 | 636 | 0.5354a* | 55.62 | 714 | 0.6027a** | 51.43 |
| | Anth | 145.56~624.93 | 361.33 | | | | | | |
| | Caro | 27.71~56.26 | 39.65 | | | | | | |
| | Chla / Chlb | 2.27~2.73 | 2.51 | | | | | | |
| | Total Chl /Anth | 0.81~1.13 | 0.96 | | | | | | |
| | Total Chl /Caro | 5.96~8.99 | 8.78 | | | | | | |
| Purple leaf flowering cherry | Chl a | 216.48~548.78 | 450.65 | 592 | 0.9388d*** | 26.64 | 716 | 0.9291fg*** | 28.68 |
| | Chl b | 80.81~201.41 | 164.59 | 580 | 0.9207cd*** | 11.14 | 714 | 0.8750cde*** | 13.99 |
| | Total Chl | 297.29~750.20 | 615.24 | 590 | 0.9339d*** | 37.79 | 714 | 0.9170ef*** | 42.37 |
| | Anth | 157.17~297.25 | 232.40 | | | | | | |
| | Caro | 121.31~215.97 | 187.42 | | | | | | |
| | Chla / Chlb | 2.55~2.92 | 2.73 | | | | | | |
| | Total Chl /Anth | 1.89~2.52 | 2.65 | | | | | | |
| | Total Chl /Caro | 2.45~3.47 | 3.28 | | | | | | |

[z]Poplar = *Populus trichocarpa* x *P. deltoids* 'UCC-1'; Apple = *Malus domestica* 'Fuji'; Almond = *Prunus dulcis* 'Nonpareil', Purple leaf flowering cherry = *Prunus blireiana*; Purple leaf plum = *Prunus cerasifera* 'Newport'

[y]Pigment and the ratio between the concentration of leaf Chl a and Chl b (Chla / Chlb); the ratio between total Chl and Anth (Total Chl /Anth); the ratio between total Chl and Caro (Total Chl / Caro).

[x]The pigment concentration range from low to high among the samples tested and the mean within the same genotype

[w]$OW_{Chl}$ = optimum wavelength for Chl assessment determined by regression

[v]$R^2$ and RSME from regression of reflectance values on Chl concentration; * $p<0.05$, ** $p<0.01$, *** $p<0.001$; $R^2$ followed by the same letters for same Chl of the same genotype within the same row are not significantly different ($p<0.05$, Fisher's Z-Test, n=72)

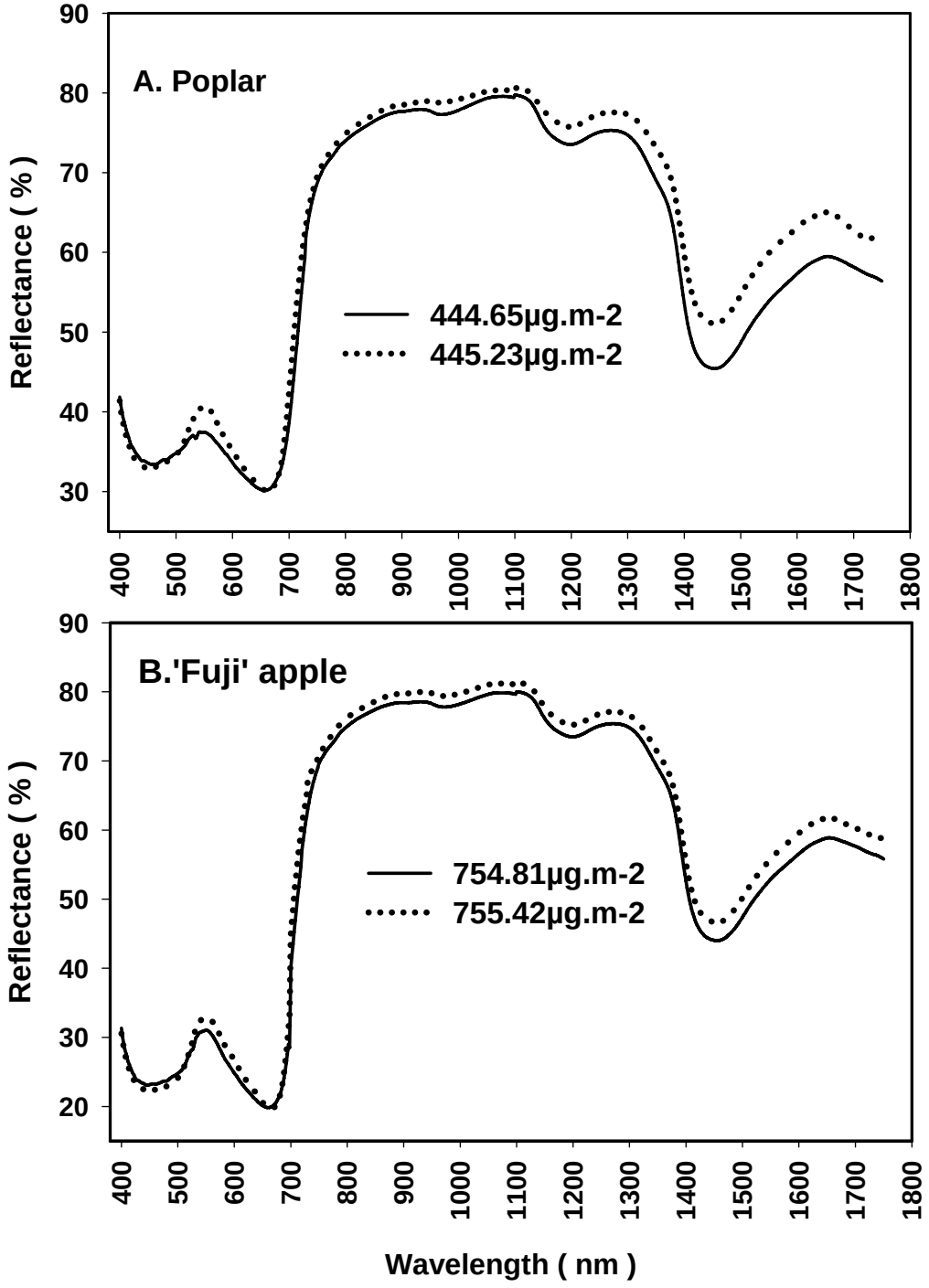


Figure 5.1 Reflectance spectra from two poplar (*Populus trichocarpa* × *P. deltoides*) leaves (A) and two apple (*Malus domestica* 'Fuji') leaves (B) with similar concentrations of total chlorophyll. Each line represents the reflectance value from scanning one leaf disc from 400 nm to 2500 nm at 2 nm intervals.

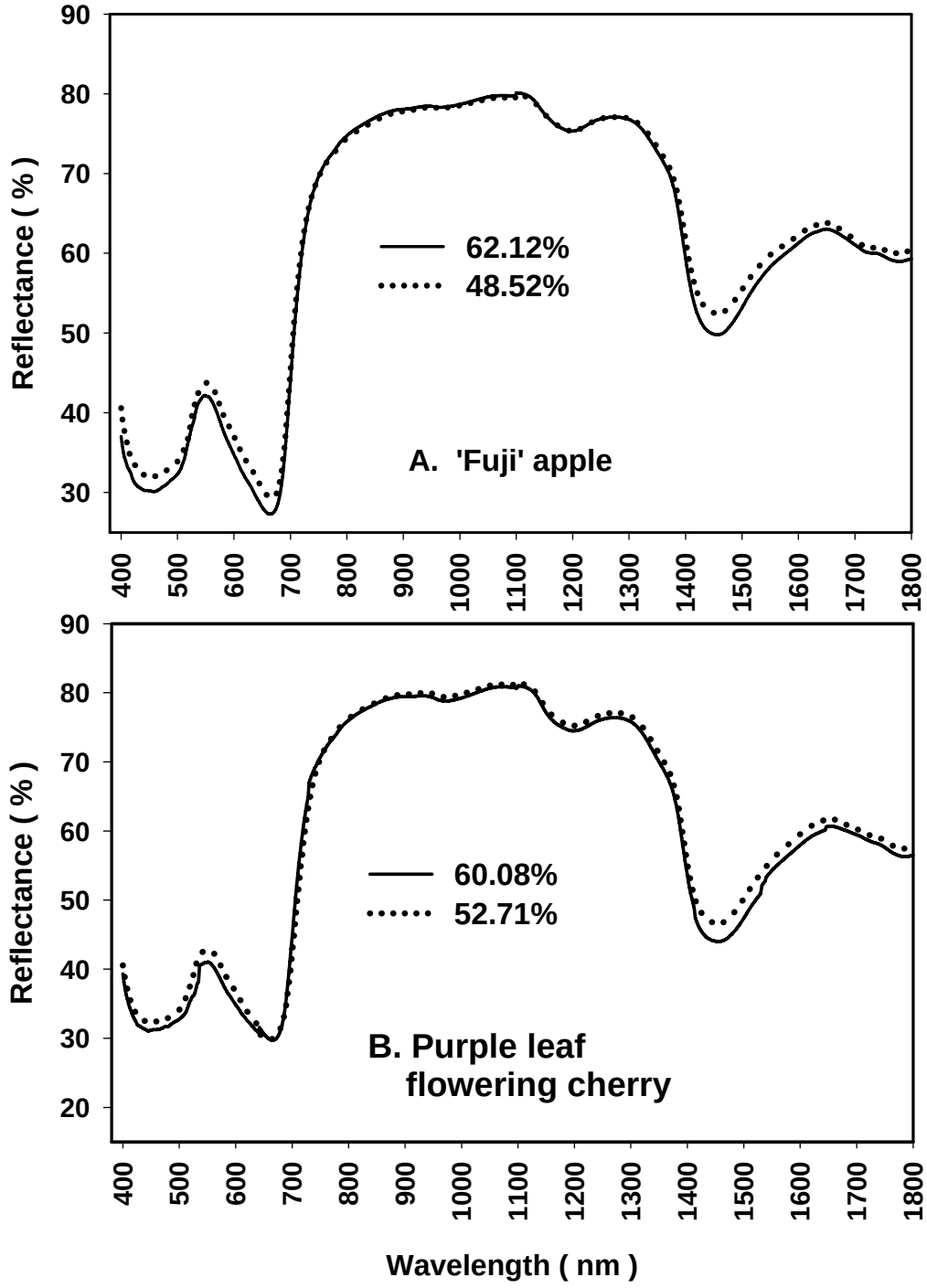


Figure 5.2 Reflectance spectra from the same leaf of (A) 'Fuji' apple (*Malus domestica* 'Fuji') and (B) purple leaf flowering cherry (*Prunus blireiana*) with different water status (% water based on fresh weight). Each line represents the mean reflectance value of 6 leaves before and after dehydration from 400 nm to 2500 nm at 2 nm intervals.

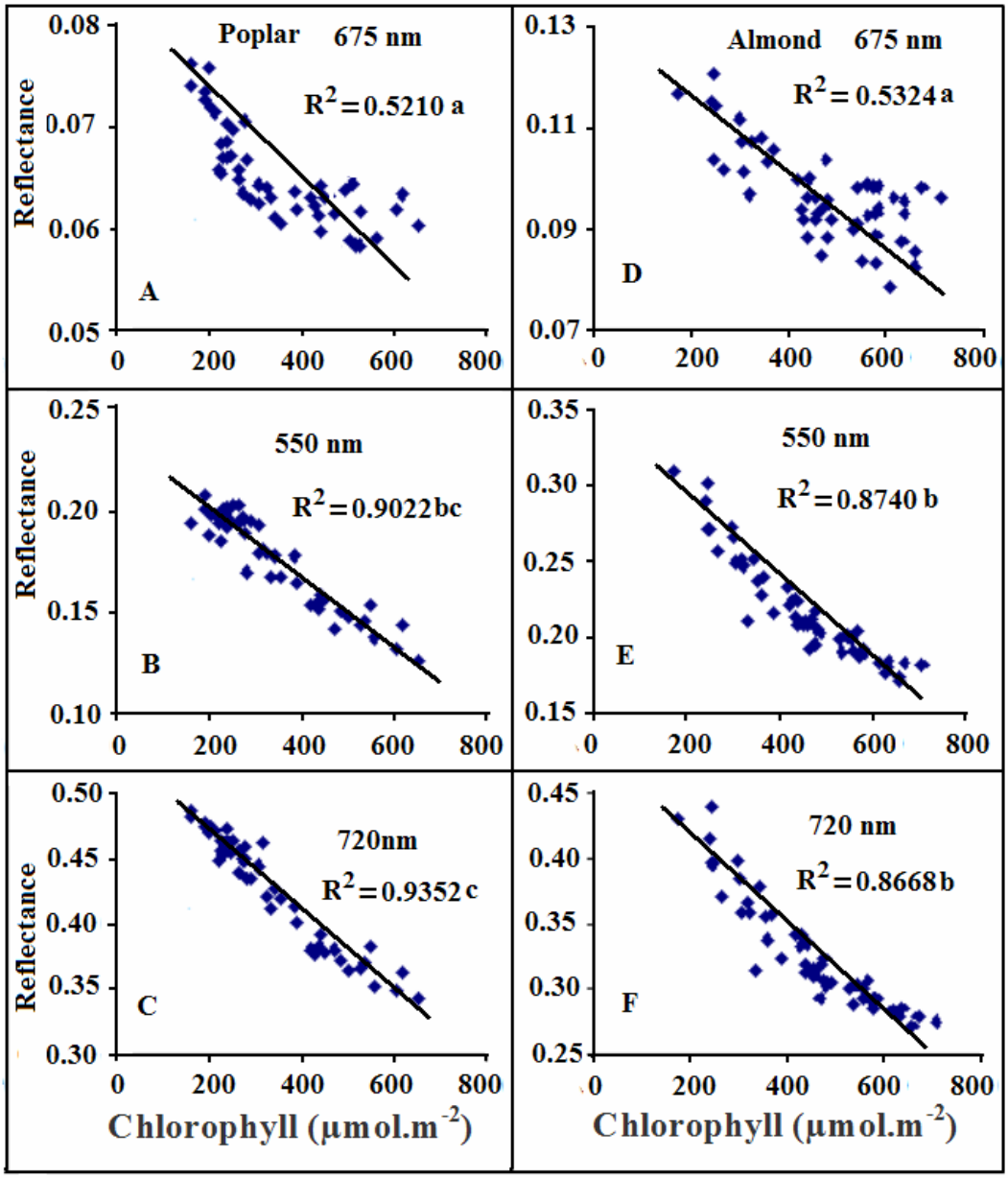


Figure 5.3 Relationships between reflectance at 550 nm, 675, nm and 720 nm wavelengths and total chlorophyll (Chl) concentrations in the leaves of 'Fuji' apple (A-C), poplar (D-F) and almond (G-I). $R^2$ values followed by the same letter not significantly different ($p<0.05$, Fisher's Z-Test, n=60)

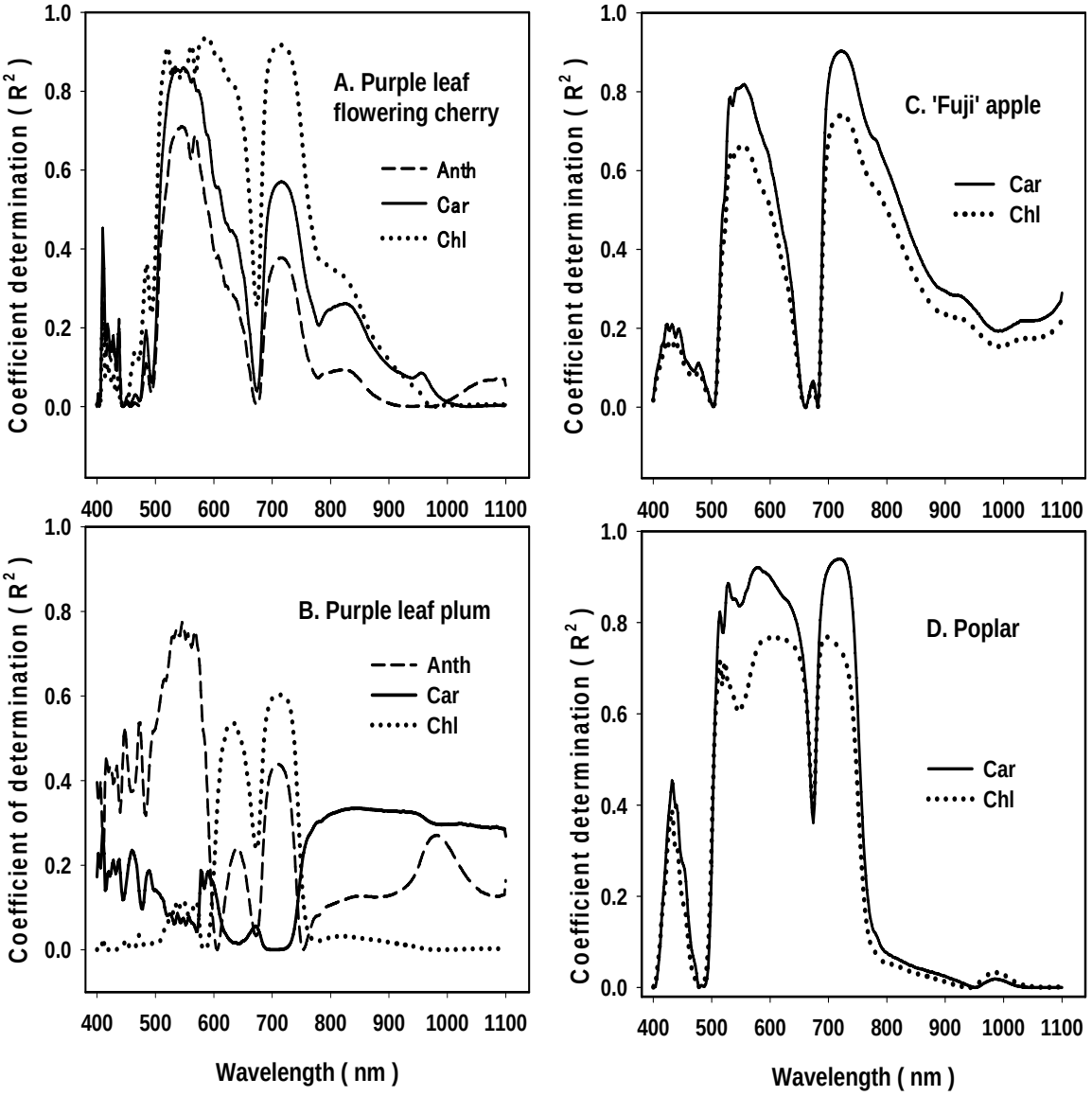


Figure 5.4. The coefficients of determination ($R^2$) for the relationship between the spectral reflectance at 1 nm intervals from 300 nm to 1100 nm and pigment concentrations in leaf discs from (A) purple leaf flowering cherry, (B) 'Fuji' apple, (C) purple leaf plum, and (D) poplar. Anth = anthocyanin; Car = carotenoids; Chl = chlorophyll. Each line represents the $R^2$ value from the regression of 72 leaf samples.

# CHAPTER 6

# VARIABILITY IN ESTIMATES OF CHLOROPHYLL AND NITROGEN BY TRANSMISSION AND REFLECTANCE USING HAND-HELD METERS IS A FUNCTION OF METER PARAMETERS AND SAMPLING TECHNIQUE

## 6.1 Abstract

One-year-old 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 grown with one of six different rates of nitrogen (N) fertilizer were used to assess the influence of meter parameters and sampling technique on the precision and accuracy of commercial hand-held meters (SPAD-502, CCM-200, and CM-1000) used for estimating chlorophyll (Chl) and N in fresh leaves. Concentrations of Chl and N in leaves were determined and spectroradiometry was used to compare optimum wavelengths (OW) and algorithms for Chl and N assessment to those used by hand-held meters. Our results showed that SPAD-502 was more precise and more accurate than CCM-200 and CM-1000 for assessing Chl and N in fresh leaves. The precision and accuracy of CM-1000 output was similar to that of CCM-200 when CM-1000 measurements were taken at a constant sampling distance. However, if sampling distance was not constant, the output precision of CM-1000 significantly decreased. The Chl-sensitive wavelength used by CM-1000 (700 nm) was more accurate at estimating Chl and N concentrations than the wavelengths used by SPAD-502 (650 nm) and CCM-200 (660nm); however the 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 spectroradiometery on leaves from the same plants, we found the most accurate wavelengths for Chl and N assessment occurred within two regions of the visible spectrum: visible (540-580 nm) and red edge (700-730 nm). However, N assessment using these wavelengths was less accurate than Chl assessment. Using transmission or reflectance with one OW in either the visible or red edge region for Chl and N assessment was more accurate than or similar to using any of the wavelengths employed by the hand-held meters we tested. When an OW was used with a NIR wavelength (750-1000 nm) in the form of simple ratio or any other algorithm to compensate for leaf texture, we found accuracy of Chl and N assessment was greater than the accuracy achieved using only one wavelength. Use of a NIR wavelength was especially important if the accuracy of the estimate obtained using either the Chl- or N-sensitive wavelength alone was small (e.g. $R^2$<0.8000 for Chl or $R^2$<0.6000 for N). We also found that OW in both visible and red edge regions varied among species, but this variation did not influence the accuracy of Chl and N assessment. Three meter prototypes were developed based on the OW and indices we identified and the factors influencing commercial accuracy we found. Our results indicate that the Prototype-III, using Chl-sensitive OW with a Chl-insensitive NIR wavelength in combination with the meter design of constant light source and precision sampling distance, was more accurate than all commercial hand-held meters for Chl assessment and than the CM-1000 for N assessment across all species we tested.

## 6.2 Introduction

Nitrogen (N) is the macroelement most frequently limiting growth and productivity of non-leguminous plants (Below 1995, Meisinger 1984). Excess application

of N may lead to contamination of ground and surface water while low N-availability can result in reduced yield and profit (Bullock and Anderson 1998). Efficient management of N to achieve an optimum productivity while preserving environmental quality is an important objective in modern agricultural systems and requires frequent plant and soil testing to ensure that neither too much nor too little N is applied. The standard methods for assessing N-status in plants are destructive and time consuming (Handson and Shelley 1993).

Most N in leaves is found in chlorophyll (Chl) and leaf N concentration is closely related to leaf Chl concentration and photosynthetic capacity (Evans 1983, Seemann et al. 1987, Syvertsen 1987, Uchida et al. 1982; Yoshida and Coronel 1976). Chl concentration is an indirect measure of N-status (Filella et al. 1995, Moran et al. 2000) and a sensitive indicator of various stresses; however traditional methods for quantifying Chl concentrations require time-consuming wet chemical methods in solvent extraction. Recently, nondestructive hand-held meters (e.g. SPAD-502, Minolta Corp., Japan; CCM-200 Chl Content Meter, Opti-Science, Inc., Tyngsboro, MA; CM 1000 Chl Meter, Spectrum Technologies, Inc., Plainfield, IL) have been developed to estimate relative leaf Chl and N concentrations, based on principles of light transmission or reflectance. These meters provide output in the form of relative numbers (index values) that are positively correlated with leaf Chl and N concentration in a wide variety of plant genotypes include annual, perennial and woody plants (Bullock and Anderson 1998, Costa et al. 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 the output from these hand-held meters vary among meters even though all use two wavelengths, one Chl-sensitive

wavelength and one Chl-insensitive wavelength (reference wavelength, RW), to assess leaf Chl and N (Markwell et al. 1995, Minolta 1989, Opti-Science 2000, Whaley 2001).

Compared to hand-held meters, which just use two wavelengths and yield a single index value, portable reflectometers can measure reflectance across the entire spectrum from ultraviolet, visible to near-infrared (NIR) wavelengths (Curran et al.1990, Adams et al. 1999, Datt 1999, Gamon & Surfus 1999); and spectroradiometers can measure both reflectance and transmittance of the entire spectrum (Schepers et al. 1996). By including wavelengths across the entire spectrum when assessing Chl, researchers can determine an almost infinite number of indices with different transformations and obtain more accurate and sensitive results (Richardson et al. 2002). Moreover, complete spectral analysis provides the ability to select and evaluate the optimum wavelengths for Chl assessment ($OW_{Chl}$) and develop indices for assessing Chl and other pigments (Adams et al. 1999, Lichtenthaler et al. 1966, Merzlyak et al. 2003).

Many researchers have studied the effect of genotype (Bullock and Anderson 1998, Peng et al. 1993, Schepers et al. 1992, Sunderman and Lamm 1991, Takebe and Yoneyama 1989), leaf developmental stage (Neilsen et al. 1995, Peng et al. 1993, Piekielek and Fox 1992), 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), water status ( Martinez and Guiamet 2004) and other plant characteristics on the accuracy of the transmission-based SPAD-502 (Markwell et al. 1995). Two studies have discussed how leaf characteristics influence meter accuracy by altering leaf light scatter, reflection, absorption and transmission properties (Monje and Bugbee 1992, Markwell et al. 1995). However, few studies have

characterized how meter parameters (i.e. meter wavelength, the consistency and constancy of sampling distance and light source, etc) influence the variability in meter accuracy. Richardson et al. (2002) compared two hand-held transmission-based meters (SPAD-502 and CCM-200) with the reflectance indices developed for canopy-level remote sensing and concluded that relative Chl concentration was more accurately estimated by reflectance rather than transmission. However, the wavelengths used in their reflectance indices were different from those used in hand-held meters. Therefore, differences in the accuracy of Chl assessment may have been a result of differences in wavelengths rather than difference in methods (reflectance vs. transmission).

Our prior work (Chapter 3 and Chapter 4) has shown that wavelengths and indices used for Chl assessment are important parameters for determining assessment accuracy. However, no research has been published that compares how parameters used by different meters and sampling methods affect the accuracy of Chl and N assessment. The objectives of this study were to (1) characterize the influence of different meter parameters on their accuracy of Chl and N assessment; (2) identify how sampling factors and leaf characteristics alter the accuracy of meters that use different wavelengths; and (3) develop a hand-held meter with better accuracy for non-destructive assessing Chl and N in fresh leaves.

## 6.3 Materials and methods

### *6.3.1 Plant materials*

'Nonpareil' almond (*Prunus dulcis* (Mill.) D.A. Webb), poplar (UCC-1, a hybrid of *Populus trichocarpa* × *P. deltoides*, Union Camp, Princeton, NJ) and bench-grafted

Fuji apple (*Malus domestica* Borkh) trees 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-10$P_2O_5$-20$K_2O$ water-soluble fertilizer with micronutrients (Plantex Corp.,Ontario, Canada). When the new shoots were approximately 15 cm long, plants were moved to full sunlight and fertilized weekly with Plantex® for 3 weeks. Beginning from 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.

### *6.3.2 Comparison of parameters and output from hand-held meters*

Three commercially available hand-held Chl meters were evaluated: SPAD-502 (Minolta Corp., Japan), CCM-200 Chl Content Meter (Opti-Science, Inc., Tyngsboro, MA), and CM 1000 Chl Meter (Spectrum Technologies, Inc., Plainfield, IL). 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 and 940 nm. The claimed accuracy of the SPAD-502 is ±1.0 SPAD units. The CCM-200weighs 180 g, has a 0.71$cm^2$ measurement area, and calculates a Chl content index (CCI) based on absorbance measurements at 660 and 940 nm. The claimed accuracy of the CCM-200 is ±1.0 CCI units. The CM-1000 weights 692g and calculates an index in CM-1000 units based on reflectance at 700 and 840 nm. The recommended sampling distance is from 28.4 to183.0 cm corresponded with the sampling scope of 1.10 to18.8 cm in diameter that is outlined with the high powered lasers. All

instruments were calibrated before measurements by following the procedures recommended by the manufacturers.

The variability of the SPAD-502, CCM-200 and CM-1000 in assessing Chl and N was determined using leaves from 'Fuji' apple grown with one of three different rates of N fertilizer. Each leaf was measured 24 times with the SPAD-502 and CCM-200, by closing the sampling head with different pressure to measure the variation of sampling distance on the precision of the meter output. The precision of the CM-1000 for each leaf was measured 24 times at a constant sampling distance of 50 cm. After measurement, leaves were removed from trees and Chl and N concentrations were determined. To compare the accuracy among different meters, readings and indices were transformed into Chl and N concentrations based on Chl and N concentration values per unit reading or Index. The standard deviations and standard errors of the output values from different meters were analyzed and used to assess meter precision and accuracy. Statistical analyses were performed with NCSS-2004 Statistical System Software (NCSS Statistical Analysis Software, Kaysville, UT).

### *6.3.3 Leaf transmittance and reflectance measurement*

At the end of August, 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 and transmittance of leaf discs were determined from 300 to 1100 nm at 1 nm intervals using Li-1800 spectroradiometer (Li-Cor Inc., Lincoln, NE). Two scans were made per sample and then averaged. The transmittance or

reflectance spectrum for each scan was calculated as $T_\lambda$(or $R_\lambda$) = (leaf radiance at wavelength λ)/(transmittance (or reflectance) standard radiance at wavelength λ).

### *6.3.4 Accuracy of the CM-1000 under different sampling conditions*

The influence of sampling distance on the accuracy of the CM-1000 was assessed by determining the CM-1000 Index of leaves from poplar trees grown with one of three different rates of N fertilizer. Measurements were taken at 10 cm intervals with target leaves 30 to 90cm from the meter. For every 10 cm interval each leaf was measured 10 times and the mean of these measurements calculated for each distance. After measurement, leaves were removed from trees and Chl and N concentrations were determined.

The influence of shading on the accuracy of the CM-1000 was assessed by determining the CM-1000 Index of individual poplar leaves under four light exposure conditions: (1) ambient light sensors and target leaf in full sunlight; (2) ambient light sensors in full sunlight while target leaf in shade; (3) ambient light sensors and target leaf in shade; and (4) ambient light sensors in shade while target leaf in full sunlight. Ten measurements were taken on the same leaf under each of the different shading conditions and the mean of these measurements calculated for each light exposure condition. After measurement, leaves were removed from trees and Chl and N concentrations were determined.

The influence of measurement orientation in relation to incident radiation on the accuracy of the CM-1000 was assessed by determining the CM-1000 Index of individual poplar leaves at 9:00 am with incident radiation coming from the east using four

measurement orientations: (1) west, (2) south, (3) north, and (4) east. Ten measurements were taken on the same leaf at each of the different orientations and the mean of these measurements calculated for each orientation. After measurement, leaves Chl and N concentrations were determined.

ANOVA and Tukey-Kramer Multiple-Comparison tests were used to analyze the effect of sampling distance, shading and measurement orientation on CM-100 accuracy.

### *6.3.5 Chlorophyll and N analysis*

Discs (1 $cm^2$) were excised from leaves with a cork borer. The leaf discs were cut into small 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). N concentration of the remaining portion of each leaf was determined by the Kjeldahl procedure (Horneck et al. 1989).

### *6.3.6 Regression analyses of spectral reflectance, transmission, Chl and N data*

Using Microsoft Visual Basic 6.0 (Microsoft Corp., Redmond, WA), custom software was developed to directly perform simple linear regression equations (linear-least-squares-fit) and calculate root mean square error (RMSE) and coefficients of determination ($R^2$) between the spectral reflectance reading at each 1 nm wavelength interval from 300 nm to 1100 nm and Chl concentrations in leaf discs. The $R^2$ of the reflectance vs. Chl relationship 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 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. Similar regression analyses were performed between the transmission values and Chl concentrations. Linear regression equations, $R^2$-curves, and RSME curves were also developed for the relationship between reflectance and transmission readings and N concentrations to predict optimal wavelengths for N assessment ($OW_N$). Pearson correlations among regressions were examined with the significance levels of $p<0.05$, $p<0.01$ and $p<0.001$. Difference between $R^2$ was tested as pairwise comparison using Fisher's Z-test (Lawley 1938 ).

### *6.3.7 Leaf transmittance and reflectance indices calculations*

Transmittance and reflectance values at 650 nm, 660nm, 700 nm, 840 nm, 940 nm and the OW ($OW_{Chl}$ and $OW_N$) selected for almond, poplar and 'Fuji' apple were transformed by using three published indices recommended to compare the algorithms used for assessment of foliar Chl and N concentrations: (1) two single wavelengths simple ratio (SR), (2) normalized difference vegetation index (NDVI), and (3) reflectance integral index (RII) (Gitelson & Merzlyak, 1994).

A SR is also called the vegetation index (VI) if the ratio is between the NIR region and red region wavelengths (Richardson et al. 2002). The SR in our study was calculated as the ratio of reflectance of $OW_{Chl}$ or $OW_N$ we selected for the different genotypes in the

visible (540-580nm) and red edge (700-730nm) regions of the spectrum divided by the reflectance of a NIR wavelength (750 – 1100 nm, e.g. 940nm) that is related to leaf texture (e.g. $R_{540-580}/R_{940}$; $R_{700-730}/R_{940}$; see Chapter 5).

A normalized difference vegetation index (NDVI) is a standard index used in canopy-level remote sensing and is calculated as $(R_{NIR}-R_{red})/(R_{NIR}+R_{red})$, here $R_{NIR}$ is the reflectance in the NIR region of the spectrum and $R_{red}$ is the reflectance in the red region (Gamon et al. 1995; Gamon and Qiu 1999, Peñuelas & Filella 1998, Richardson et al. 2002). The revised version of the NDVI is called Chl Normalized difference index (Chl NDI) calculated as Chl NDI = $(R_{750} – R_{705})/(R_{750} + R_{705})$, which is more highly correlated with leaf Chl concentrations (Richardson et al. 2002, Gitelson and Merzlyak 1994). The Chl NDI used in our study was developed using the $OW_{Chl}$ or $OW_N$ we selected for the different genotypes in the visible region and red edge of the spectrum in combination of the $OW_{Chl}$ with a NIR wavelength [e.g. $(R_{940}-R_{540-580})/(R_{940}+R_{540-580})$, $(R_{940}-R_{700-730})/(R_{940}+R_{700-730})$].

RII is calculated using a discrete summation approximation to the following integral: $RII = \int_{705}^{750}(R_\lambda / R_{710} -)d\lambda$. In our study we calculated both reflectance integral index (RII) and transmission integral index (TII) using transmission or reflectance from 700 to 740 nm divided by wavelength 710nm: $RII = \int_{700}^{740}(R_\lambda / R_{710} -)d\lambda$ and $TII = \int_{700}^{740}(R_\lambda / R_{710} -)d\lambda$.

### *6.3.8 Development of meter prototypes*

Three meter prototypes were developed using internal light-emitting diodes (LEDs) and constant sampling distance in our study: (1) Prototype-I measures

transmission of two Chl-related wavelengths (560 nm in green and 700 nm in red edge) and calculates output based on single wavelength index; (2) Prototype-II measures transmission of two Chl-related wavelengths (560 nm and 700 nm), one texture related wavelength in the NIR (940 nm) and one water-related wavelength in the MIR (1450 nm) and calculates output based on a simple ratio (SR) index ($T_{940}/T_{700}$ or $T_{940}/T_{560}$); (3) Prototype-III was similar to Prototype-II plus a GPS receiver and a HP iPAQ PDA-5555 for assessing and saving GPS positioning information.

### *6.3.9 Accuracy comparison among meter prototypes and commercial meters*

Linear regressions between meter readings and leaf Chl or N concentrations were developed for meter prototypes and commercial meters to compare meter accuracy based on using the coefficient of determination ($R^2$) and root mean square error (RSME) of different meters. Fisher's Z-Test was used to compare the $R^2$ difference (Lawley 1938).

## 6.4 Results

### *6.4.1 Meter variability in Chl and N assessment*

Meter output values increased with increasing Chl and N concentrations in leaves and varied between measurements on the same leaves (Table 6.1). The repeatability and precision of SPAD-502 measurements was greater (smaller standard error for transformed Chl and N results) than the CCM-200 and CM-1000 output (Table 6.2, Table-6-3). The precision of CCM-200 and CM-1000 were similar when measurements of CM-1000 were based on a constant sampling distance.

### *6.4.2 Relationships among output values from different meters*

There were close curvilinear relationships among the output values from the different meters (Figure 6.1). The relationship between output values from the SPAD-502 and the CCM-200 had higher $R^2$-values than the relationship between output values from the SPAD-502 and CM-1000 or between CCM-200 and CM-1000.

### *6.4.3 Comparison of wavelengths used by hand-held meters for Chl and N assessments*

The wavelengths of CM-1000 (700 nm) and the OW ($OW_{Chl}$ and $OW_N$) we identified had similar accuracy (e.g. the $R^2$ and RMSE), and were more accurate (e.g. higher $R^2$ and lower RMSE) for predicting Chl and N concentrations using either transmission or reflectance than the wavelengths used in the SPAD-502 (650 nm) or CCM-200 (660 nm) (Table 6.4, Table 6.5). The wavelengths of the two transmission based hand-held meters are very similar and wavelengths used by both meters showed similar accuracy for estimating Chl and N using either transmission or reflectance..

### *6.4.4 Accuracy of the CM-1000 under different sampling conditions*

Within the manufacturers' recommended range of sampling distance (28.4 to 183 cm), output values from the CM-1000 decreased with increasing distance from target sample (50 cm to 90 cm, $p<0.05$, Figure 6.2) and the influence of measurement distance on meter output increased with increasing leaf Chl concentration (Figure 6.2). Output values from the CM-1000 when used either the ambient light sensor or the target leaf in shade were lower than when the sensor and the target leaf were both in full sun (Figure 6.3). Both the light brightness (BRT) recorded by the ambient sensor and output values

from the CM-1000 meter were significantly affected by the measurement orientation to incident radiation (Figure 6.4).

### *6.4.5 Optimum wavelengths for Chl and N assessments*

$R^2$-curves based on the relationships between Chl (or N) concentrations and transmission or reflectance values for all genotypes showed two peaks with large $R^2$ and small RMSE, one in the visible region (540-580 nm) and one in the red edge region (700-730 nm) of the spectrum (Figure 6.5, Figure 6.6, Figure 6.7, Figure 6.8). The peak in the $R^2$-curve for transmission in the visible region was wider than the peak of the $R^2$-curve for reflectance. Wavelengths with large $R^2$ values in combination with small RMSE values indicate that these wavelengths in the visible and red edge regions of the spectrum could be used for both $OW_{Chl}$ and $OW_N$. However, the N peaks on the $R^2$-curves based on N concentrations were much smaller than the peaks on the $R^2$-curves based on Chl, indicating that these wavelengths are more accurate for Chl assessment than N assessment. Different species had different $OW_{Chl}$ and $OW_N$ with different $R^2$ and RMSE; however, the $OW_{Chl}$ or $OW_N$ for the different species tested were in two relative narrow wavebands in the visible (540-580 nm) and red edge regions (700-730 nm) of the spectrum (Figure 6.5, Figure 6.6, Figure 6.7, Figure 6.8)

### *6.4.6 Comparison of regression indices based on OW and wavelengths used by hand-held meters*

Among the three hand-held meters, the SPAD-502 was the most accurate (e.g. highest $R^2$ and smallest RMSE) for measuring Chl in 'Fuji' apple (Table 6.6) and for measuring N in poplar and almond (Table 6.7). Using a constant sampling distance, the

accuracy of indices based on wavelengths used by CM-1000 and CCM-200 were similar. Wavelengths and the indices used by the hand-held meters were generally less accurate than the indices developed by using the $OW_{Chl}$ or $OW_N$ selected using a spectroradiometer. The indices using a single $OW_{Chl}$ or $OW_N$ from either the visible or red edge of the spectrum were similar to or more accurate than the hand-held meters using two wavelengths (one Chl-sensitive wavelength 650, 660 or 700nm; and the other Chl-insensitive wavelength 840 or 940 nm). The indices using two wavelengths in a simple ratio (e.g. $T_{940}/T_{Chl5XX}$ or $T_{940}/T_{Chl7XX}$, $R_{940}$/or $R_{940}/R_{Chl7XX}$) or indices with one Chl-sensitive $OW_{Chl}$ or $OW_N$ in combination with one NIR wavelength (i.e. 940 nm) increased the accuracy of Chl and N assessment compared to the hand-held meters. If the accuracy of the estimate obtained using a single wavelength was high (e.g. $R^2 > 0.9000$ for Chl or $R^2 > 0.6000$ for N), addition of the NIR wavelength to the algorithm did not improve $R^2$.

### *6.4.7 Comparison of meter accuracy*

The $R^2$ and RMSE values from the regression between Chl and N concentrations and meter output showed that Prototype-I, using only Chl-related $OW_{Chl}$ (560 nm in green or 700 nm in red edge), had higher accuracy (larger $R^2$ and smaller RMSE) than the CM-1000 across all species for both N and Chl assessment. The Prototype-II was more accurate than the CM-1000 and the CCM-200 for Chl assessment and than the CM-1000 for N assessment across all species tested. Prototype-III was more accurate than all hand-held meters for Chl assessment and than the CM-1000 for N assessment across all species. Among three commercial hand-held meters, SPAD-502 and CCM-200 were more

accurate than CM-1000. All the meters tested had higher accuracy in assessing Chl than assessing N (Table 6.8 and Table 6.9).

## 6.5 Discussion

When light hits a leaf, it can be reflected, scattered or re-emitted, absorbed, or transmitted (Fukshansky 1981, Kirk 1994, Richardson et al. 2002). Because the function of Chl pigments are to absorb quanta of incident light, some researchers have hypothesized that instruments that use absorbance to estimate Chl concentrations (e.g. SPAD-502 and CCM-200) are better than instruments that rely on reflectance measures. Richardson et al (2002) determined that the relative Chl concentration of leaves was better estimated using reflectance rather than by absorbance; however the wavelengths they used in their reflectance indices were different from those used in the hand-held meters. Moreover, both SPAD-502 (Markwell et al 1995) and CCM-200 actually measure transmission rather than directly measure adsorption, although the owner's manuals for both SPAD-502 and CCM-200 state their measurement is based on absorbance (Minolta, 1989; OptiScience, 2000). Our results indicated that the accuracy ($R^2$ and RMSE) of Chl and N assessment was influenced by the Chl- and N-related wavelengths used in the meters or indices and not by measurment method (i.e. transmission or reflectance) (Figure 6.5, Figure 6.6, Figure 6.7, Figure 6.8). Both transmission and reflectance values had similar accuracy for estimating Chl or N when $OW_{Chl}$ or $OW_N$ were used in indices (Table 6.4, Table 6.5, Table 6.6, Table 6.7).

The Chl-related wavelength used in CM-1000 was more accurate than the Chl-related wavelength used in SPAD-502 or CCM-200 (Table 6.4, Table 6.5). The CM-1000, however,

was less accurate than SPAD-502 and CCM-200 (Table 6.2, Table 6.3), indicating that, in addition to Chl related wavelengths, other factors are also very important to meter accuracy (e.g. sampling distance and light source).

We found that variation in light intensity due to shading and orientation to incident radiation are very important factors to meter accuracy. Both SPAD-502 and CCM-200 have two internal light-emitting diodes (LEDs) that produce red light at 650 nm (SPAD-502) or 660 nm (CCM-200) and NIR light at 940 nm. The wavelengths in the red region are sensitive to differences in leaf Chl concentrations, whereas the NIR wavelength is sensitive to difference in leaf texture (Minolta, 1989; OptiScience, 2000). CM-1000, instead of internal LEDs, uses outside natural lights at wavelengths of 700 nm and 840 nm to estimate the quantity of Chl in plant leaves (Whaley, 2001). Chl absorbs 700 nm light and, as a result, the reflection of that wavelength from the leaf is reduced compared to the reflected 840 nm light. The 840 nm light is unaffected by leaf Chl concentration and serves as a parameter to compensate for leaf physical differences. SPAD-502 and CCM-200 use inside LEDs to keep the measurement light source constant, while the CM-1000 uses an uncontrolled light source (natural sunlight) for measurement and thus output can vary based on changes in light. The ambient light sensor and sample target sensor on the CM-1000 are not located closely to each other, thus, differences in incident light on the sensors can result in overestimation or underestimation of Chl estimates (Figure 6.3, Figure 6.4). This is one reason that CM-1000 is not as accurate as the SPAD-502 or CCM-200.

We also found that the accuracy of output from handheld meters is influenced by sampling distance. For example, the recommended sampling distance for the CM-1000 is

28.4 to 183 cm. Within this recommended range, we found estimates obtained on the same leaf decreased with increasing distance between the target leaf and the meter (Figure 6.2). In the indices used by all the hand-held meters we tested, an increase in sampling distance decreases the light intensity received by the photodiode and thus variation in sampling distance decreases meter accuracy. Our results indicated that SPAD-502 had less variation in sampling distance than CCM-200., plus the 650 nm wavelength used by SPAD-502 is a better wavelength for Chl assessment than the 660 nm wavelength used by CCM-200. However, according to our results, the wavelengths used by both SPAD-502 (650 nm) and CCM-200 (660 nm) are in the red region and not the OW ($OW_{Chl}$ and/or $OW_N$).

Our results show that one of the most important factors influencing the accuracy of hand-held meters for estimating Chl and N is whether the Chl- and N-related wavelengths used in the meter are the $OW_{Chl}$ or $OW_N$. Although the 650 nm wavelength used by SPAD-502 and the 660 nm wavelength used by CCM-200 are optimum for assessment of extracted Chl in solvent, these wavelengths were not the OW for nondestructive Chl or N assessment (Figure 6.5, Figure 6.6, Figure 6.7, Figure 6.8, Table 6.4, Table 6.5). Extracted Chl has two absorbance peaks, one in the blue (400- 450 nm) the other in red (600- 700 nm). In 80% acetone solution, Chl a and Chl b can be measured by using 663.2 nm and 646.8 nm wavelengths, respectively, and total Chl is the sum of Ch a and Ch b (Lichtenthaler and Wellburn 1983). Most Chl in plant leaves is in the form of Chl a; therefore, total Chl in extracted solution can also be directly measured by using 650 or 660 nm wavelengths (Lichtenthaler 1987). This might be the reason why SPAD-502 and CCM-200 use 650nm and 660nm wavelengths to assess Chl in plant leaves. However, for nondestructive assessment of Chl in leaves, the $OW_{Chl}$ is very different

from that of extracted Chl in solvent (Gitelson et al.2003). In nondestructive measurement of fresh leaves, the absorption coefficients of Chl in the red range 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 low amounts of Chl are sufficient to saturate absorption. When Chl exceeds 150$\mu g.m^{-2}$, total absorption reaches a maximum, and any increase in Chl concentration does not cause an increase in total absorption (Gitelson et al.2003). Our result further confirmed this hypothesis.

According to our results, the wavelengths in visible (540 - 580 nm) and red edge (700 - 730 nm) regions of the spectrum are the most accurate wavebands (highest $R^2$ and lowest RMSE) for assessment of Chl and N in 'Fuji' apple, poplar and almond using either transmission or reflectance (Figure 6.5, Figure 6.6, Figure 6.7, Figure 6.8, Table 6.4, Table 6.5). Large $R^2$ with small RMSE indicated that the wavelengths in the visible and red edge regions can be used as $OW_{Chl}$ or $OW_N$ for Chl and N assessment. In the visible and red edge regions, the specific absorption coefficient of extracted Chl in solvent (like acetone) is very low; it does not exceed 6% of that of the remainder of the visible region of the spectrum (Heath 1969, Lichtenthaler 1987); however, fresh green leaves absorb more than 80% of incident light in the visible green and red edge regions (Gausman and Allen 1973, Gitelson and Merzlyak 1994). Wavelengths from these regions penetrate four- to six-fold deeper into leaves than wavelengths from the remainder of the visible region of the spectrum (Fukshansky et al. 1993, Merzlyak and Gitelson 1995). Therefore, absorption of light is high enough from wavelengths in the visible and red edge regions to provide high sensitivity of transmission or reflectance

values in relation to Chl assessment (Figure 6.5, Figure 6.6, Figure 6.7, Figure 6.8, Table 6.4, Table 6.5). In addition to high sensitivity, absorption of wavelengths from the visible and red edge regions by Chl is not saturated by leaf Chl concentrations from 1 to 1200 $\mu g.m^{-2}$, whereas 650 nm and 660 nm wavelengths are saturated by leaf concentrations less than 150 $\mu g.m^{-2}$ (Gitelson et al.2003).

In plant leaves, N concentration is closely related to Chl concentration. Our results indicated that the wavelengths in the visible and red edge regions that were sensitive to Chl can also be used to successfully assess leaf N concentration (Figure 6.5, Figure 6.6, Figure 6.7, Figure 6.8, Table 6.4, Table 6.5). Different species (e.g. 'Fuji' apple, poplar or almond) have different $OW_{Chl}$ or $OW_N$ (Table 6.4, Table 6.5). We found that these differences are relatively small and were found in two narrow wavebands in the visible (560±20nm) and red edge (715±15nm) regions of the spectrum. Thus it is possible to select "common" OW in these regions that are sensitive to Chl and N and one NIR wavelength (750-1100 nm) that is insensitive to Chl but sensitive to leaf texture to develop some "common" indices for assessing Chl and N across genotypes.

In addition to the importance of using OW in Chl and N assessment, selection of a superior index is also very important to meter accuracy. We found that using either transmission or reflectance with a single OW from either the visible region or red edge for estimating Chl and N is more accurate than or similar to using the parameters or methods employed by any of the hand-held meters (Table 6.4, Table 6.5, Table 6.6, Table 6.7); however, if the OW is combined with one NIR wavelength in the form of simple ratio or any other algorithm to compensate for leaf texture, accuracy of Chl and N assessment can be increased (Table 6.6, Table 6.7). This is especially important if the $R^2$ of the single

wavelength index-based regression is less than 0.8000 for Chl or 0.6000 for N caused by either the influence of leaf parameters, not using $OW_{Chl}$ and $OW_N$ or other factors. This result verified the limitation of single wavelength indices in Chl and N assessment.

## 6.6 Conclusion

We believe the accuracy of current hand-held meters for assessing Chl and N in leaves could be greatly improved if meters used OW from either the visible (560±20nm) or red edge (715±15nm) regions of the spectrum in combination with one Chl- and N-insensitive NIR wavelength (750-1100 nm) and superior indices in the form of SR, NDVI, RII or any other indices. Meter accuracy and precision could also be increased if a consistent light source (internal LEDs) and constant sampling distance could be employed in developing new meters. The meter Prototype-III developed based on the above information was more accurate than all commercial hand-held meters for Chl assessment and than the CM-1000 for N assessment across all species we tested.

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Table 6.1 Variability in output values obtained from 'Fuji' apple leaves with different chlorophyll (Chl) and nitrogen (N) concentrations using three hand-held meters

| Meter[z] | Leaf Chl ($\mu mol.m^{-2}$) | Leaf N (%) | Meter Output[y] | | | |
|---|---|---|---|---|---|---|
| | | | Range | Mean | SD | SE |
| SPAD-502 | 378.30 | 1.5870 | 38.60-39.00 | 38.84b | 0.13 | 0.04 |
| CM-200 | 378.30 | 1.5870 | 24.50-29.00 | 26.96a | 1.65 | 0.41 |
| CCM-1000 | 378.30 | 1.5870 | 172.00-196.00 | 185.23c | 7.68 | 2.05 |
| SPAD-502 | 515.62 | 1.9731 | 46.00-47.00 | 46.51a | 0.40 | 0.10 |
| CM-200 | 515.62 | 1.9731 | 49.60-55.20 | 52.56b | 2.27 | 0.57 |
| CCM-1000 | 515.62 | 1.9731 | 192.00-221.00 | 204.64c | 9.42 | 3.81 |
| SPAD-502 | 708.06 | 2.3874 | 55.70-57.00 | 56.29a | 0.40 | 0.13 |
| CM-200 | 708.06 | 2.3874 | 73.10-85.90 | 81.12b | 4.44 | 1.28 |
| CCM-1000 | 708.06 | 2.3874 | 211.00-247.00 | 231.25c | 12.78 | 3.80 |

[z] SPAD-502 (Minolta Corp., Japan), CCM-200 (Opti-Science, Inc., Tyngsboro, MA), CM-1000 (Spectrum Technologies, Inc., Plainfield, IL).

[y]Range, mean, standard deviation (SD) and standard error (SE) of 24 readings per leaf. Means within the save leaf sample follower by the same letter are not significantly different ($p<0.05$, LSD test).

Table 6.2 Variability in estimated chlorophyll (Chl) concentrations of 'Fuji' apple leaves with different Chl concentrations obtained using three hand-held meters

| Meter[z] | Leaf Chl ($\mu mol.m^{-2}$) | Chl/unit Index[y] | Chl estimates ($\mu mol.m^{-2}$) [x] | | | |
|---|---|---|---|---|---|---|
| | | | Range | Mean | SD | SE |
| SPAD-502 | 378.30 | 9.70 | 375.95-379.84 | 377.90a | 1.28 | 0.37 |
| CM-200 | 378.30 | 13.04 | 343.83-406.98 | 375.41a | 23.13 | 5.78 |
| CCM-1000 | 378.30 | 1.93 | 351.28-400.30 | 375.79a | 15.69 | 4.19 |
| SPAD | 515.62 | 10.92 | 509.94-523.25 | 516.60a | 4.45 | 1.11 |
| CM-200 | 515.62 | 9.34 | 486.56-541.50 | 514.03a | 22.28 | 5.57 |
| CCM-1000 | 515.62 | 2.33 | 483.77-556.84 | 520.31a | 23.74 | 7.33 |
| SPAD-502 | 708.06 | 12.42 | 700.64-716.99 | 708.82a | 5.08 | 1.61 |
| CM-200 | 708.06 | 8.24 | 638.09-749.82 | 693.96a | 38.72 | 11.18 |
| CCM-1000 | 708.06 | 2.87 | 646.06-756.29 | 701.18a | 39.13 | 11.64 |

[z] SPAD-502 (Minolta Corp., Japan), CCM-200 (Opti-Science, Inc., Tyngsboro, MA), CM-1000 (Spectrum Technologies, Inc., Plainfield, IL).

[y]Chl/unit Index calculated by dividing the Chl concentration by the meter Mean output

[x] Range, mean, standard deviation (SD) and standard error (SE) of estimated Chl values based of 24 readings per leaf. Means within the save leaf sample follower by the same letter are not significantly different ($p<0.05$, LSD test).

Table 6.3 Variability in estimated nitrogen (N) concentrations of 'Fuji' apple leaves with different N concentrations obtained using three hand-held meters.

| Meter[z] | N (%) | N/Unit Index[y] | N estimates (%) [x] | | | |
|---|---|---|---|---|---|---|
| | | | Range | Mean | SD | SE |
| SPAD-502 | 1.5870 | 0.0409 | 1.5771-1.5934 | 1.5853a | 0.0054 | 0.0015 |
| CM-200 | 1.5870 | 0.0589 | 1.4424-1.7073 | 1.5749a | 0.0970 | 0.0243 |
| CCM-1000 | 1.5870 | 0.0086 | 1.4736-1.6792 | 1.5764a | 0.0658 | 0.0176 |
| SPAD-502 | 1.9731 | 0.0424 | 1.9514-2.0023 | 1.9769a | 0.0170 | 0.0043 |
| CM-200 | 1.9731 | 0.0375 | 1.8619-2.0721 | 1.9670a | 0.0853 | 0.0213 |
| CCM-1000 | 1.9731 | 0.0096 | 1.8512-2.1308 | 1.9910a | 0.0909 | 0.0280 |
| SPAD-502 | 2.3874 | 0.0424 | 2.3624-2.4175 | 2.3900a | 0.0171 | 0.0054 |
| CM-200 | 2.3874 | 0.0294 | 2.1514-2.5282 | 2.3398a | 0.1306 | 0.0377 |
| CCM-1000 | 2.3874 | 0.0103 | 2.1783-2.5500 | 2.3642a | 0.1319 | 0.0392 |

[z] SPAD-502 (Minolta Corp., Japan), CCM-200 (Opti-Science, Inc., Tyngsboro, MA), CM-1000 (Spectrum Technologies, Inc., Plainfield, IL).

[y]N/unit Index calculated by dividing N concentration by the meter Mean output

[x] Range, mean, standard deviation (SD) and standard error (SE) of estimated N values based of 24 readings per leaf. Means within the save leaf sample follower by the same letter are not significantly different ($p<0.05$, LSD test).

Table 6.4 Correlation coefficients ($R^2$) and root mean square errors (RSME) of different wavelengths for estimating chlorophyll (Chl) in leaves of three plant species by transmission and reflectance

| Plant Species[z] | Wavelength Source[y] | Wavelength (nm) | Transmission | | Reflectance | |
|---|---|---|---|---|---|---|
| | | | $R^{2\,x}$ | RMSE ($\mu mol.m^{-2}$)[x] | $R^{2\,x}$ | RMSE ($\mu mol.m^{-2}$)[x] |
| Poplar | $OW_{Chl}$ | 575 | -- | -- | 0.9166bc$^{***}$ | 38.23 |
| | $OW_{Chl}$ | 580 | 0.9357c$^{***}$ | 30.38 | -- | -- |
| | SPAD-502 | 650 | 0.8768b$^{***}$ | 34.26 | 0.7604a$^{**}$ | 88.81 |
| | CCM-200 | 660 | 0.7921a$^{**}$ | 40.43 | 0.7001a$^{**}$ | 93.11 |
| | CM-1000 | 700 | 0.9271bc$^{***}$ | 35.23 | 0.8903b$^{***}$ | 44.97 |
| | $OW_{Chl}$ | 704 | 0.9294c$^{***}$ | 33.49 | -- | -- |
| | $OW_{Chl}$ | 720 | -- | -- | 0.9352c$^{***}$ | 33.48 |
| Apple | $OW_{Chl}$ | 552 | 0.8844b$^{***}$ | 87.00 | 0.7696c$^{***}$ | 122.07 |
| | SPAD-502 | 650 | 0.7830a$^{***}$ | 119.18 | 0.1242b$^{ns}$ | 237.96 |
| | CCM-200 | 660 | 0.7860a$^{***}$ | 118.34 | 0.0048a$^{ns}$ | 253.67 |
| | CM-1000 | 700 | 0.9071b$^{***}$ | 77.97 | 0.8131c$^{***}$ | 109.94 |
| | $OW_{Chl}$ | 712 | 0.9144b$^{***}$ | 74.86 | | |
| | $OW_{Chl}$ | 717 | -- | -- | 0.9073d$^{***}$ | 77.41 |
| Almond | $OW_{Chl}$ | 550 | -- | -- | 0.8737b$^{***}$ | 47.55 |
| | $OW_{Chl}$ | 567 | 0.7977b$^{***}$ | 60.17 | -- | -- |
| | SPAD-502 | 650 | 0.6025a$^{**}$ | 84.36 | 0.6957a$^{**}$ | 73.81 |
| | CCM-200 | 660 | 0.4944a$^{*}$ | 95.14 | 0.6332a$^{**}$ | 81.03 |
| | CM-1000 | 700 | 0.8225b$^{***}$ | 56.37 | 0.8414b$^{***}$ | 53.28 |
| | $OW_{Chl}$ | 705 | 0.8309b$^{***}$ | 55.03 | -- | -- |
| | $OW_{Chl}$ | 710 | -- | -- | 0.8668b$^{***}$ | 48.83 |

[z]Poplar = *Populus trichocarpa* x *P. deltoids* 'UCC-1'; Apple = *Malus domestica* 'Fuji'; Almond = *Prunus dulcis* 'Nonpareil'

[y]$OW_{Chl}$ = optimum wavelength for Chl assessment determined by spectroradiometer using simple linear regression $R^2$ and RSME; and wavelengths used by SPAD-502 (650 nm), CCM-200 (660 nm) and CCM-1000 (700 nm).

[x]$R^2$ and RSME from simple linear regression of total chlorophyll concentration and reflectance or transmission values for each wavelength determined by using spectroradiometery; ns – not significant ($p>0.05$), * $p<0.05$, **$p<0.01$, $p<0.001$; $R^2$ followed by the same letter for same genotype are not significantly different ($p<0.05$, Fisher's Z-Test; n=72)

Table 6.5 Correlation coefficients ($R^2$) and root mean square errors (RSME) of different wavelengths for estimating nitrogen (N) in leaves of three plant species by transmission and reflectance

| Plant Species[z] | Wavelength Source[y] | Wavelength (nm) | Transmission | | Reflectance | |
|---|---|---|---|---|---|---|
| | | | $R^2$ [x] | RMSE (%) [x] | $R^2$ [x] | RMSE (%) [x] |
| Poplar | $OW_N$ | 576 | -- | -- | 0.9542bc*** | 0.0877 |
| | $OW_N$ | 585 | 0.9133b*** | 0.1206 | -- | -- |
| | SPAD-502 | 650 | 0.8633ab*** | 0.1515 | 0.8040a*** | 0.1814 |
| | CCM-200 | 660 | 0.8414a*** | 0.1632 | 0.7425a** | 0.2081 |
| | CM-1000 | 700 | 0.9066ab*** | 0.1252 | 0.9332b*** | 0.1059 |
| | $OW_N$ | 703 | 0.9077b*** | 0.1245 | -- | -- |
| | $OW_N$ | 720 | -- | -- | 0.9735c*** | 0.0667 |
| Apple | $OW_N$ | 550 | 0.6663a** | 0.3871 | 0.6117c** | 0.4148 |
| | SPAD-502 | 650 | 0.5889b** | 0.4296 | 0.0948b ns | 0.6334 |
| | CCM-200 | 660 | 0.5810b** | 0.4339 | 0.0029a ns | 0.6648 |
| | CM-1000 | 700 | 0.6660a** | 0.3873 | 0.6292c** | 0.4054 |
| | $OW_N$ | 728 | -- | -- | 0.7057c** | 0.3612 |
| Almond | $OW_N$ | 558 | -- | -- | 0.4963b** | 0.5360 |
| | $OW_N$ | 566 | 0.4103a** | 0.5800 | -- | -- |
| | SPAD | 650 | 0.3097b* | 0.6275 | 0.3807a* | 0.5943 |
| | CCM-200 | 660 | 0.2488b* | 0.6545 | 0.3380a* | 0.6144 |
| | CM-1000 | 700 | 0.4748a** | 0.5473 | 0.4748 b** | 0.5473 |
| | $OW_N$ | 705 | 0.4949a** | 0.5367 | -- | -- |
| | $OW_N$ | 713 | -- | -- | 0.5028b** | 0.5325 |

[z]Poplar = *Populus trichocarpa* x *P. deltoids* 'UCC-1'; Apple = *Malus domestica* 'Fuji'; Almond = *Prunus dulcis* 'Nonpareil'

[y]$OW_N$ = optimum wavelength for N assessment determined by spectroradiometer using simple linear regression $R^2$ and RSME; and corresponding wavelengths used by SPAD-502 (650 nm), CCM-200 (660 nm) and CCM-1000 (700 nm).

[x]$R^2$ and RSME from simple linear regression of nitrogen concentration and reflectance or transmission determined for each wavelength by using spectroradiometery; ns – not significant ($p>0.05$), * $p<0.05$, ** $p<0.01$, *** $p<0.001$; $R^2$ followed by the same letters for same genotype are not significantly different ($p<0.05$, Fisher's Z-Test, n=72)

Table 6.6 Correlation coefficients ($R^2$) and root mean square errors (RSME) from hand-held meters and optimum wavelength ($OW_{Chl}$) related indices for estimating chlorophyll (Chl) concentrations in leaves of poplar, apple, and almond.

| Method[z] | Poplar[y] | | Apple | | Almond | |
|---|---|---|---|---|---|---|
| | $R^{2w}$ | RMSE[w] ($\mu mol.m^{-2}$) | $R^{2w}$ | RMSE[w] ($\mu mol.m^{-2}$) | $R^{2w}$ | RMSE[w] ($\mu mol.m^{-2}$) |
| SPAD-502 | 0.9011 ab*** | 43.08 | 0.9078cdef*** | 76.64 | 0.7927ab** | 65.74 |
| CM-1000 | 0.8623 a*** | 53.83 | 0.8028a*** | 119.17 | 0.6860a** | 89.25 |
| CCM-200 | 0.8823 ab*** | 48.29 | 0.8232ab*** | 110.91 | 0.6842a** | 89.65 |
| $T_{Chl5XX}$ | 0.9222bcde*** | 37.23 | 0.9346efghi*** | 65.80 | 0.8235bc*** | 56.23 |
| $T_{Chl7XX}$ | 0.9102abc*** | 40.56 | 0.9183cdefg*** | 72.39 | 0.8542bc*** | 51.08 |
| $T_{940}/T_{Chl5XX}$ | 0.9356cdef*** | 33.52 | 0.9563i*** | 57.00 | 0.8937cd*** | 43.48 |
| $T_{940}/T_{Chl7XX}$ | 0.9477def*** | 30.16 | 0.9511ghi*** | 59.11 | 0.9280de*** | 35.93 |
| $(T_{940}-T_{Chl5XX})/(T_{940}+T_{Chl5XX})$ | 0.9338cdef*** | 34.02 | 0.9520ghi*** | 58.74 | 0.8934cd*** | 43.55 |
| $(T_{940}-T_{Chl7XX})/(T_{940}+T_{Chl7XX})$ | 0.9450def*** | 30.91 | 0.9474fghi*** | 60.60 | 0.9202de*** | 37.64 |
| Chl TNDI | 0.9469def*** | 30.39 | 0.9476ghi*** | 60.52 | 0.9267de*** | 36.21 |
| TII | 0.9483ef*** | 30.00 | 0.9478ghi*** | 60.44 | 0.9319de*** | 35.07 |
| $R_{Chl5XX}$ | 0.9420cdef*** | 31.74 | 0.8630abc*** | 94.79 | 0.9173de*** | 38.95 |
| $R_{Chl7XX}$ | 0.9447def*** | 30.99 | 0.9232defgh*** | 70.41 | 0.9184de*** | 38.72 |
| $R_{940}/R_{Chl5XX}$ | 0.9432def*** | 31.41 | 0.8935bcde*** | 82.43 | 0.9350de*** | 34.09 |
| $R_{940}/R_{Chl7XX}$ | 0.9553f*** | 28.06 | 0.9451fghi*** | 61.54 | 0.9420e*** | 32.26 |
| $(R_{940}-R_{Chl5XX})/(R_{940}+R_{Chl5XX})$ | 0.9420cdef*** | 31.74 | 0.8811abcd*** | 87.46 | 0.9356de*** | 33.93 |
| $(R_{940}-R_{Chl7XX})/(R_{940}+R_{Chl7XX})$ | 0.9548ef*** | 28.20 | 0.9433fghi*** | 62.26 | 0.9268de*** | 36.24 |
| Chl RNDI | 0.9534ef*** | 28.58 | 0.9332efghi*** | 66.36 | 0.9356de*** | 33.93 |
| RII | 0.9543ef*** | 28.33 | 0.9542hi*** | 57.85 | 0.9373de*** | 33.49 |

[z] Transmission method using two wavelengths for SPAD-502 [(650 nm and 940 nm as $\log(T_{940}/T_{650})$ ,$T_{SPAD-502}$]; CCM-200 (660 nm and 940 nm as, $T_{CCM-200}$); and optimal wavelengths ($OW_{Chl}$) in the visible (poplar, 580 nm; apple, 552 nm; almond, 567 nm; $T_{Chl5XX}$) and red edge (poplar, 705 nm; apple, 712 nm; almond, 705 nm; $T_{Chl7XX}$) regions. Reflectance method using wavelengths for CCM-1000 (700 nm and 840nm as $R_{840}/R_{700}$, $R_{CM-1000}$); and $OW_{Chl}$ in the visible (poplar, 575 nm; apple, 552 nm; almond, 550 nm; $R_{Chl5XX}$) and red edge (poplar, 720 nm; apple, 717 nm; almond, 710 nm; $R_{Chl7XX}$) regions. Normalized difference vegetation indices based on reflectance (Chl RNDI = $(R_{750} - R_{705})/(R_{750} + R_{705})$;) and integral index ( $RII = \int_{700}^{740}(R_{\lambda}/R_{710}-)d\lambda$ ); and transmission methods [Chl TNDI=$(T_{750} - T_{705})/(T_{750} + T_{705})$ and $TII = \int_{700}^{740}(T_{\lambda}/T_{710}-)d\lambda$ ].

[y]Poplar = *Populus trichocarpa* x *P. deltoids* 'UCC-1'; Apple = *Malus domestica* 'Fuji'; Almond = *Prunus dulcis* 'Nonpareil'

[x]Equations best fit equation of the method for converting reflectance, transmission or index values to Chl concentration ($\mu mol.m^{-2}$)

[w]$R^2$ and RSME from regression of reflectance or transmission values on total chlorophyll concentration ($\mu mol.m^{-2}$); **p<0.01, ***p<0.001; $R^2$ followed by the same letter within the column are not significantly different (p<0.05, Fisher's Z-Test; n=72)

Table 6.7 Correlation coefficients ($R^2$) and root mean square errors (RSME) from hand-held meters and optimum wavelength ($OW_N$) related indices for estimating nitrogen (N) concentrations in leaves of poplar, apple, and almond.

| Method[z] | Poplar[y] | | 'Fuji' apple | | Almond | |
|---|---|---|---|---|---|---|
| | $R^2$ | RMSE (%) | $R^2$ | RMSE (%) | $R^2$ | RMSE (%) |
| $T_{SPAD-502}$ | 0.8902b*** | 0.0815 | 0.6637abc*** | 0.3878 | 0.4206ab* | 0.5769 |
| $R_{CM-1000}$ | 0.7598a*** | 0.0819 | 0.5716a** | 0.4372 | 0.3864a* | 0.5955 |
| $T_{CCM-200}$ | 0.7703a*** | 0.0813 | 0.5736a** | 0.4361 | 0.3867a* | 0.5953 |
| $T_{Chl5XX}$ | 0.9485cd*** | 0.0935 | 0.6765abc*** | 0.3809 | 0.4878ab** | 0.5406 |
| $T_{Chl7XX}$ | 0.9439c*** | 0.0982 | 0.6670abc*** | 0.3860 | 0.5039b** | 0.5318 |
| $T_{940}/T_{Chl5XX}$ | 0.9559cde*** | 0.0859 | 0.7246abc*** | 0.3551 | 0.5040b** | 0.5318 |
| $T_{940}/T_{Chl7XX}$ | 0.9654cde*** | 0.0761 | 0.7139abc*** | 0.3608 | 0.5190b** | 0.5237 |
| $(T_{940}-T_{Chl5XX})/(T_{940}+T_{Chl5XX})$ | 0.9564cde*** | 0.0854 | 0.6890abc*** | 0.3742 | 0.5064b** | 0.5305 |
| $(T_{940}-T_{Chl7XX})/(T_{940}+T_{Chl7XX})$ | 0.9665cde*** | 0.0750 | 0.6849abc*** | 0.3764 | 0.5118b** | 0.5276 |
| Chl TNDI | 0.9675cde*** | 0.0739 | 0.7634c*** | 0.3342 | 0.5278b** | 0.5189 |
| TII | 0.9657cde*** | 0.0758 | 0.6985abc*** | 0.3691 | 0.5236b** | 0.5212 |
| $R_{Chl5XX}$ | 0.9641cde*** | 0.0774 | 0.6678abc*** | 0.3856 | 0.5243b** | 0.5208 |
| $R_{Chl7XX}$ | 0.9742e*** | 0.0670 | 0.6142ab*** | 0.4143 | 0.5314b** | 0.5169 |
| $R_{940}/R_{Chl5XX}$ | 0.9551cde*** | 0.0867 | 0.6533abc*** | 0.3933 | 0.5341b** | 0.5155 |
| $R_{940}/R_{Chl7XX}$ | 0.9691de*** | 0.0723 | 0.6606abc*** | 0.3894 | 0.5439b** | 0.5102 |
| $(R_{940}-R_{Chl5XX})/(R_{940}+R_{Chl5XX})$ | 0.9561cde*** | 0.0857 | 0.6495abc*** | 0.3954 | 0.5368b** | 0.5140 |
| $(R_{940}-R_{Chl7XX})/(R_{940}+R_{Chl7XX})$ | 0.9693de*** | 0.0721 | 0.6776abc*** | 0.3803 | 0.5401b** | 0.5122 |
| Chl RNDI | 0.9685de*** | 0.0729 | 0.7511bc*** | 0.3408 | 0.5415b** | 0.5115 |
| RII | 0.9667cde*** | 0.0748 | 0.6540abc*** | 0.3930 | 0.5507b** | 0.5065 |

[z] Transmission method using two wavelengths for SPAD-502 [(650 nm and 940 nm as $\log(T_{940}/T_{650})$ ,$T_{SPAD-502}$]; CCM-200 (660 nm and 940 nm as, $T_{CCM-200}$); and optimal wavelengths ($OW_{Chl}$) in the visible (poplar, 575 nm; apple, 550 nm; almond, 558 nm; $T_{Chl5XX}$) and red edge (poplar, 703 nm; apple, 700 nm; almond, 705 nm; $T_{Chl7XX}$) regions. Reflectance method using wavelengths for CCM-1000 (700 nm and 840nm as $R_{840}/R_{700}$, $R_{CM-1000}$); and $OW_{Chl}$ in the visible (poplar, 575 nm; apple, 552 nm; almond, 550 nm; $R_{Chl5XX}$) and red edge (poplar, 720 nm; apple, 728 nm; almond, 713 nm; $R_{Chl7XX}$) regions. Normalized difference vegetation indices based on reflectance (Chl RNDI = $(R_{750} - R_{705})/(R_{750} + R_{705})$;) and integral index ( $RII = \int_{700}^{740}(R_{\lambda}/R_{710}-)d\lambda$ ); and transmission methods [Chl TNDI=$(T_{750} - T_{705})/(T_{750} + T_{705})$ and $TII = \int_{700}^{740}(T_{\lambda}/T_{710}-)d\lambda$ ].

[y]Poplar = *Populus trichocarpa* x *P. deltoids* 'UCC-1'; Apple = *Malus domestica* 'Fuji'; Almond = *Prunus dulcis* 'Nonpareil'

[x]Equations best fit equation of the method for converting reflectance, transmission or index values to Chl concentration (%)

[w]$R^2$ and RSME from regression of reflectance or transmission values on total chlorophyll concentration (%); **p<0.01, ***p<0.001; $R^2$ followed by the same letter within the column are not significantly different (p<0.05, Fisher's Z-Test; n=72)

Table 6.8 Correlation coefficients ($R^2$) and root mean square error (RSME) for relationships between leaf chlorophyll (Chl) concentrations and output from different hand-held meters used to estimate Chl in leaves of different genotypes

| Genotype[z] | Meters | $R^2$ [y] | RMSE ($\mu mol.m^{-2}$) [y] |
|---|---|---|---|
| 'Fuji' apple | CM-1000 | 0.7137 a* | 145.2 |
| | CCM-200 | 0.8120 ab** | 112.35 |
| | SPAD-502 | 0.8687 bcd** | 98.67 |
| | Prototype-I | 0.9002 cde** | 78.83 |
| | Prototype-II | 0.9234 de** | 74.25 |
| | Prototype-III | 0.9322 e** | 73.22 |
| 'Gala' apple | CM-1000 | 0.7145 a** | 122.12 |
| | CCM-200 | 0.8101 ab** | 97.88 |
| | SPAD-502 | 0.8596 bc** | 88.25 |
| | Prototype-I | 0.9088 cde** | 70.45 |
| | Prototype-II | 0.9132 cde** | 67.33 |
| | Prototype-III | 0.9181 de** | 62.23 |
| Poplar | CM-1000 | 0.7434 a* | 85.33 |
| | CCM-200 | 0.8741 bcd** | 62.34 |
| | SPAD-502 | 0.8917 cd** | 56.78 |
| | Prototype-I | 0.9115 cde** | 40.56 |
| | Prototype-II | 0.9301 e** | 34.86 |
| | Prototype-III | 0.9324 e** | 33.48 |

[z] 'Fuji' apple = *Malus domestica* 'Fuji'; 'Gala' apple = *Malus domestica* 'Gala'; Poplar = *Populus trichocarpa* x *P. deltoids* 'UCC-1'

[y] $R^2$ and RSME from regression of between meter output and leaf Chl concentration; * $p<0.05$, ** $p<0.01$; $R^2$ followed by the same letter within a column are not significantly different ($p<0.05$, Fisher's Z-Test)

Table 6.9 Correlation coefficients ($R^2$) and root mean square error (RSME) for relationships between leaf nitrogen (N) concentrations and outputs from different hand-held meters used to estimate N in leaves different genotypes

| Genotype[z] | Meters | $R^2$ [y] | RMSE (%) [y] |
|---|---|---|---|
| 'Fuji' apple | CM-1000 | 0.5821abc* | 0.42 |
| | CCM-200 | 0.6923 bcde** | 0.29 |
| | SPAD-502 | 0.7213 cde** | 0.18 |
| | Prototype-I | 0.7713 e** | 0.15 |
| | Prototype-II | 0.7824 e** | 0.15 |
| | Prototype-III | 0.7932 e** | 0.14 |
| 'Gala' apple | CM-1000 | 0.5434 ab* | 0.44 |
| | CCM-200 | 0.6977 bcde** | 0.22 |
| | SPAD-502 | 0.7012 bcde** | 0.18 |
| | Prototype-I | 0.7538 de** | 0.16 |
| | Prototype-II | 0.7734 e** | 0.15 |
| | Prototype-III | 0.7836 e** | 0.15 |
| Poplar | CM-1000 | 0.5214 a* | 0.38 |
| | CCM-200 | 0.6231 abcd** | 0.2 |
| | SPAD-502 | 0.7717 e** | 0.12 |
| | Prototype-I | 0.7815 e** | 0.11 |
| | Prototype-II | 0.8004 e** | 0.1 |
| | Prototype-III | 0.8086 e** | 0.09 |

[z] 'Fuji' apple = *Malus domestica* 'Fuji'; 'Gala' apple = *Malus domestica* 'Gala'; Poplar = *Populus trichocarpa* x *P. deltoids* 'UCC-1'

[y] $R^2$ and RSME from regression of between meter output and leaf N concentration; * $p<0.05$, ** $p<0.01$; $R^2$ followed by the same letters within a column are not significantly different ($p<0.05$, Fisher's Z-Test)

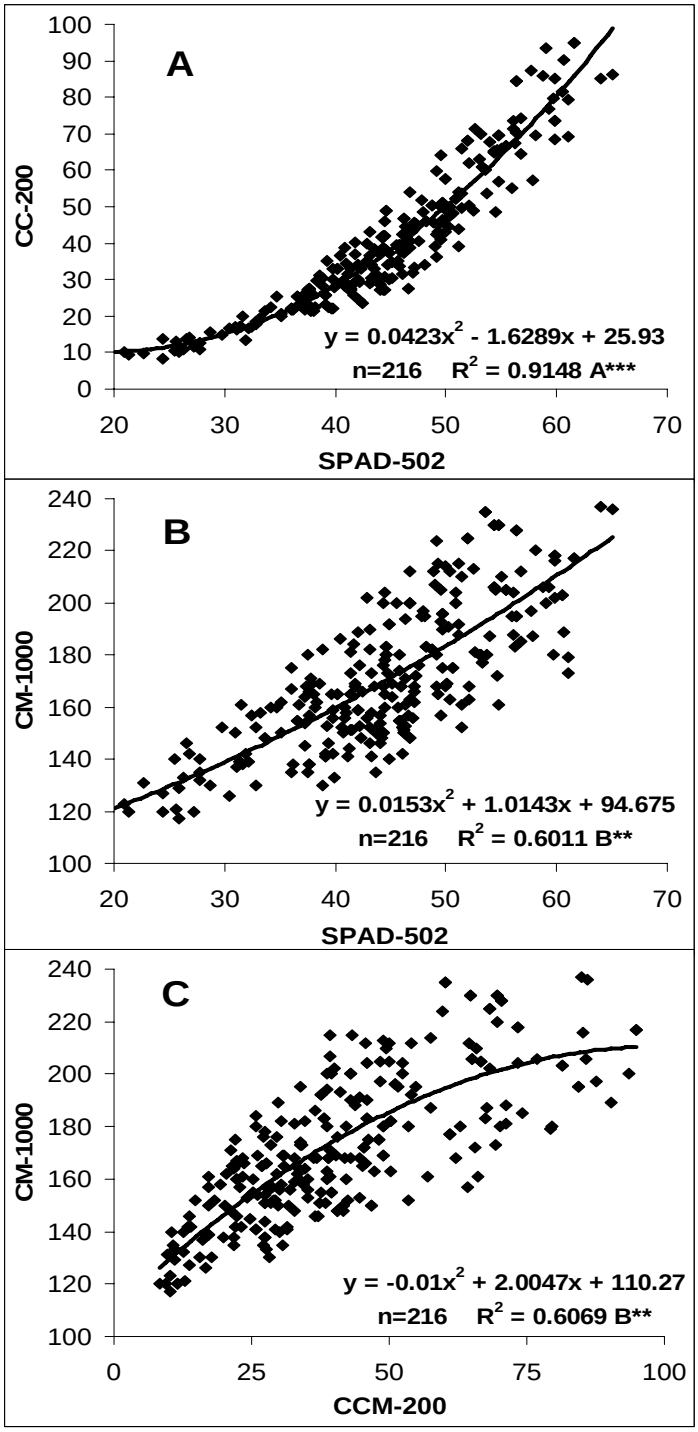


Figure 6.1 Relationships between of output values from SPAD-502, CCM-200 and CM-1000. Each data point represents the mean of 5 measurements per leaf from almond (*Prunus dulcis* 'Nonpareil'), apple (*Malus domestica* 'Fuji') and poplar (*Populus trichocarpa* × *P. deltoids*). A total of 72 leaves were measured for each genotype. **p<0.01, ***p<0.001; $R^2$ followed by the same uppercase letter are not significantly different (p<0.05, Fisher's Z-Test)

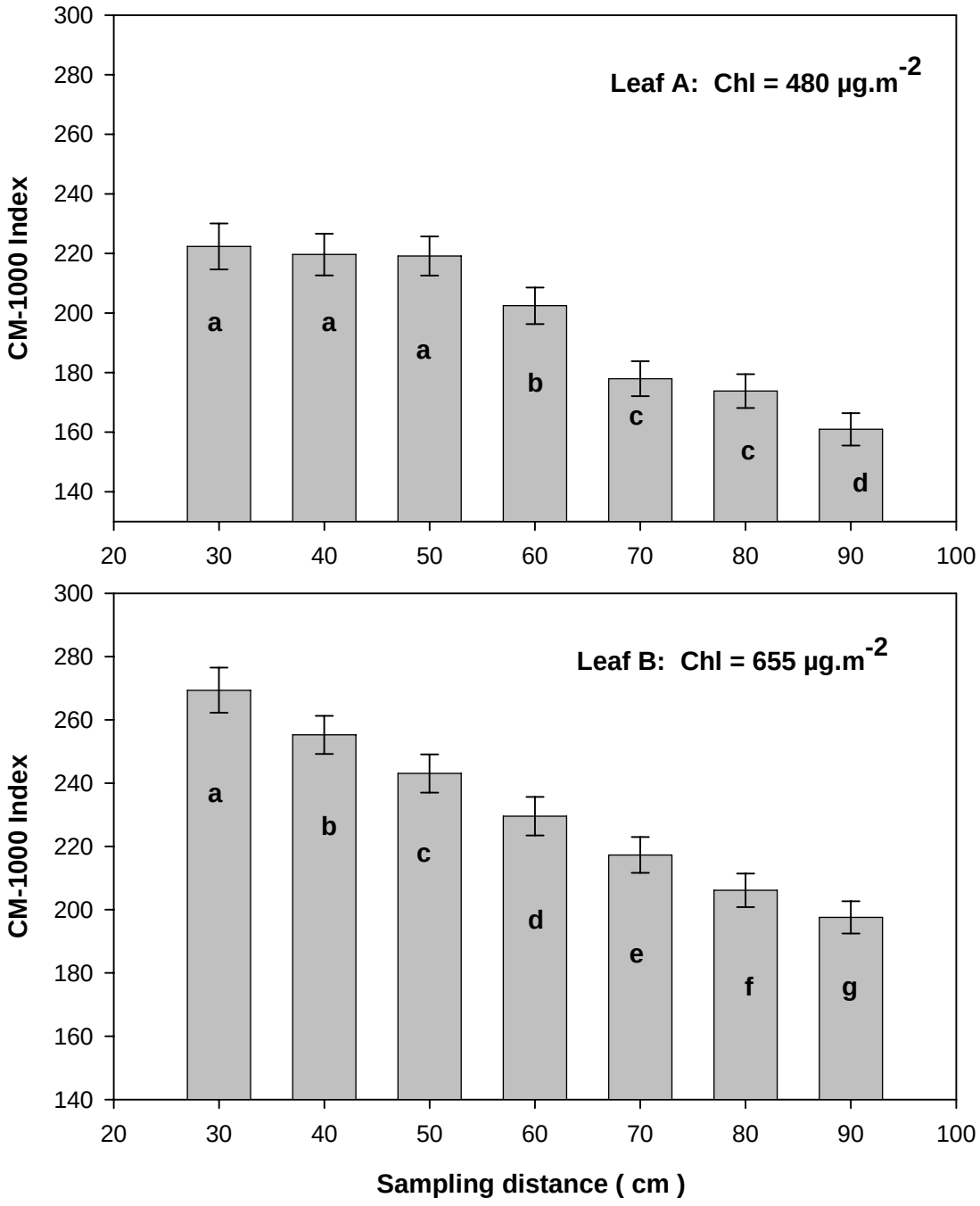


Figure 6.2 Output from a CM-1000 meter (CM-1000 Index) obtained from three poplar leaves (Leaf A and Leaf B) with different chlorophyll concentrations. Error bars are standard errors of the mean of 10 measurements per leaf. Same letters within a leaf are not significantly different ($p<0.05$, Tukey-Kramer Multiple-Comparison Test)

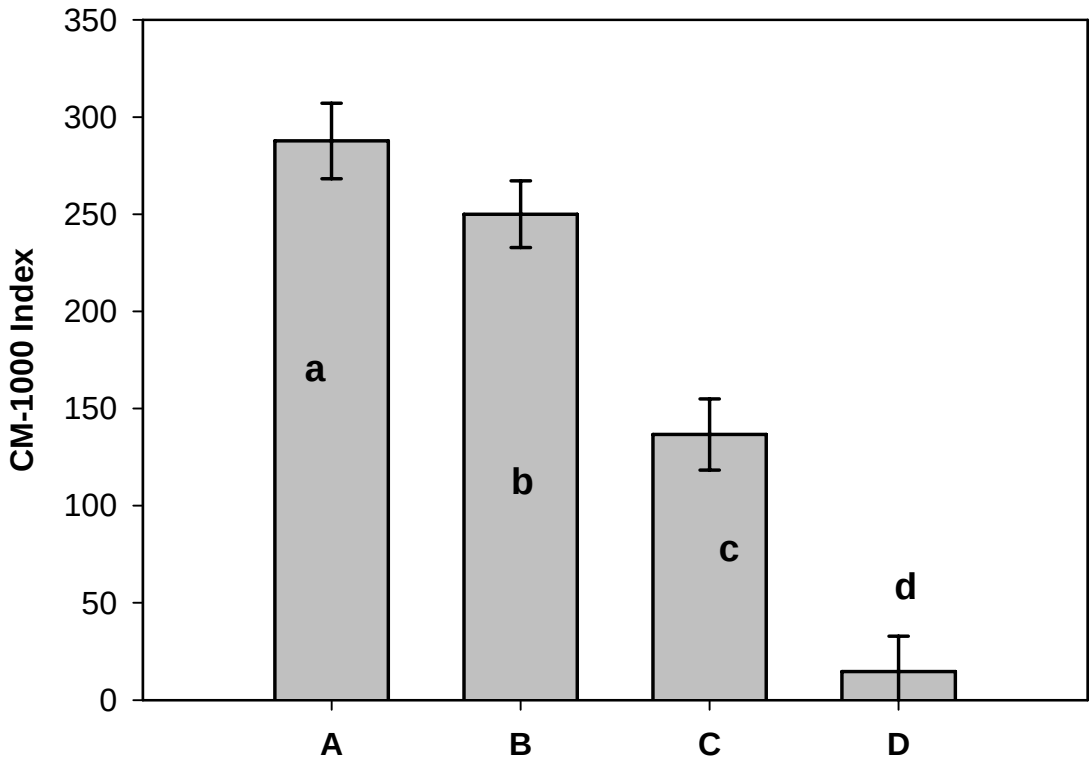


Figure 6.3 Output from a CM-1000 meter (CM-1000 Index) when ambient light sensors and target sample exposed at different light intensities. A. ambient light sensors and target leaf exposed to full sun light; light brightness BRT=4; B. ambient light sensors exposed to full sun light, target leaf in the shade, BRT=4; C. both ambient light sensors and target leaf in the shade, BRT=0; D. ambient light sensors in the shade, target leaf exposed to full sun light BRT=0. Error bars represent standard errors of a mean of 10 measurements per leaf Columns with different letters above them are significantly different (LSD test $P<0.05$; $n=10$)

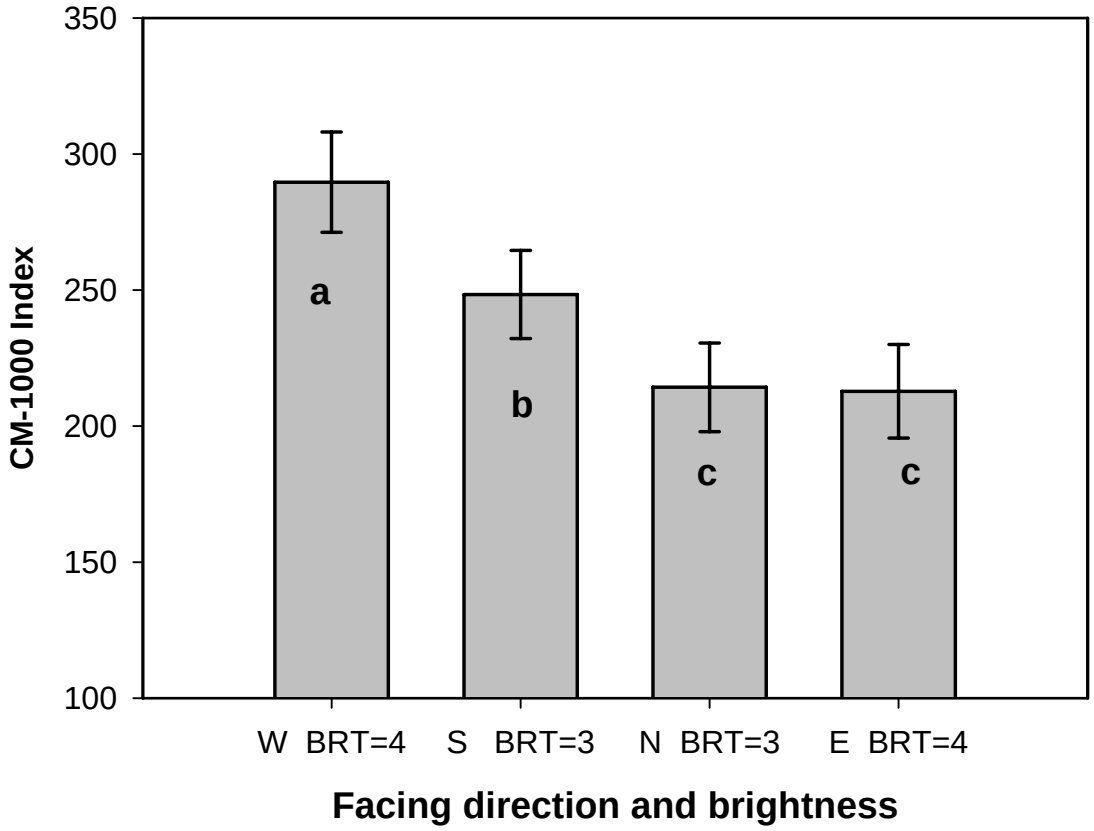


Figure 6 4 Output from a CM-1000 meter (CM-1000 Index) when measurements were taken at different orientations in relation to incident light. Index of leaves at 9:00 am with incident radiation coming from the each and measurement orientations west (W), south (S), north (N), and east (E). BRT = light brightness recorded by meter light sensors. Error bars are standard errors of a mean of 10 measurements per leaf Columns with different letters above them are significantly different (LSD test $P<0.05$; $n=10$)

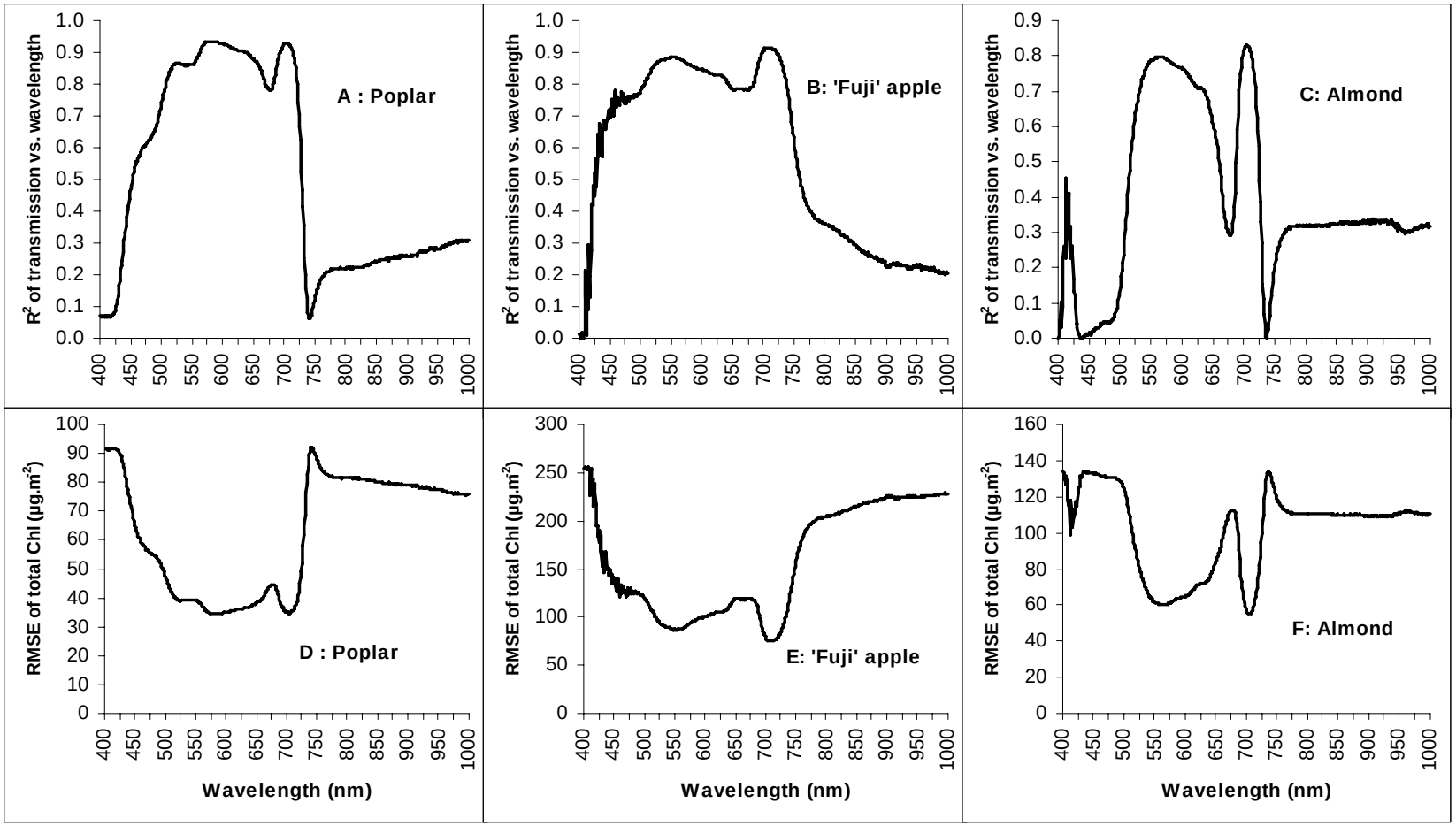


Figure 6.5 Curves of coefficients of determination ($R^2$) and root mean square errors (RMSE) for the relationships between transmission values and total chlorophyll (Chl) concentrations at 1 nm intervals from 300nm to 1100 nm in leaves of poplar (A, D), apple (B, E) and almond (C, F). Apple = *Malus domestica* 'Fuji'; Poplar = *Populus trichocarpa* x *P. deltoids*; Almond = *Prunus dulcis* 'Nonpareil'. Curves developed using simple linear regression of 72 samples per genotype.

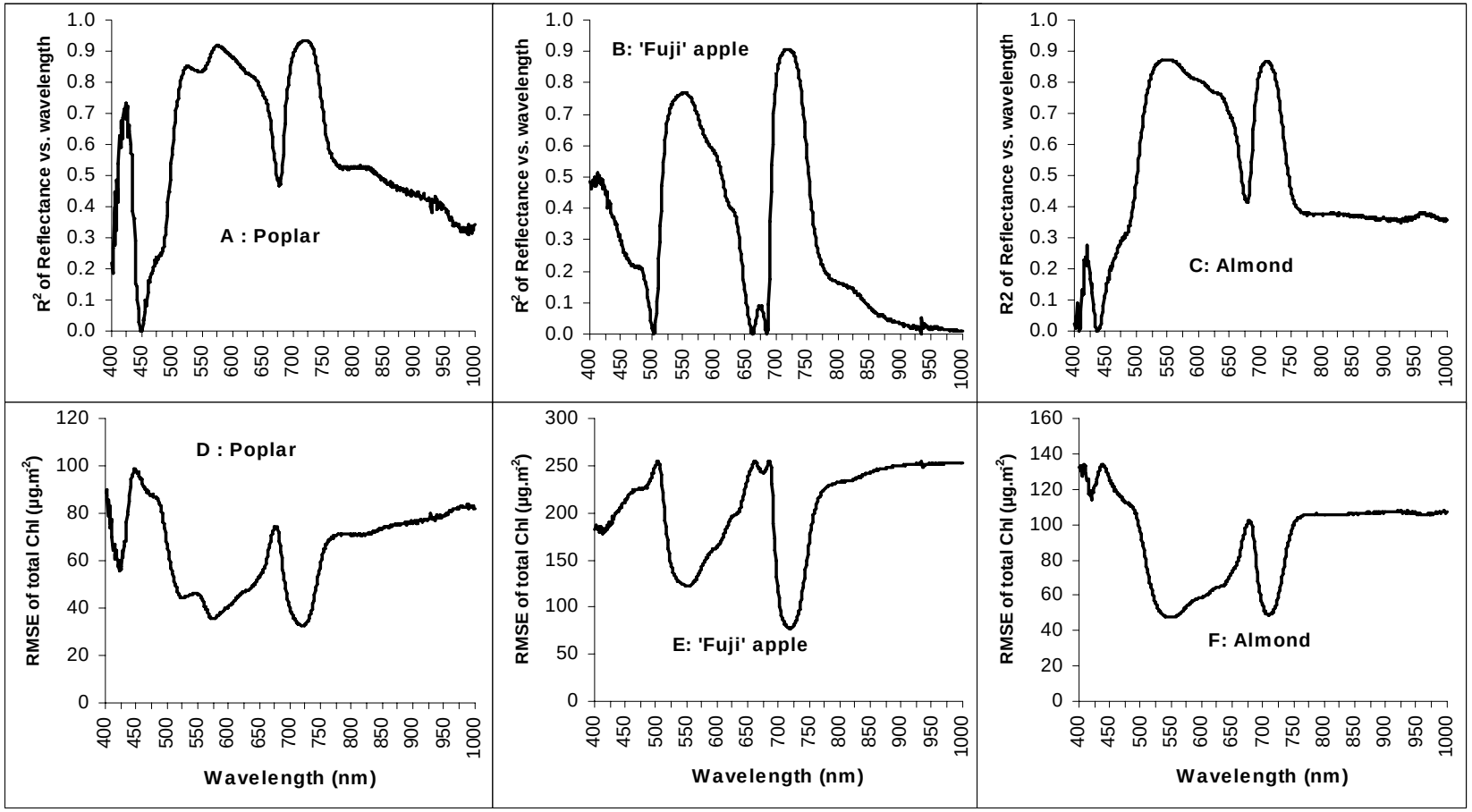


Figure 6.6 Curves of coefficients of determination ($R^2$) and root mean square error (RMSE) for the relationships between reflectance values and total chlorophyll (Chl) concentration at 1 nm intervals from 300nm to 1100 nm in leaves of poplar (A, D), apple (B, E) and almond (C, F). Apple = *Malus domestica* 'Fuji'; Poplar = *Populus trichocarpa* x *P. deltoids*; Almond = *Prunus dulcis* 'Nonpareil'. Curves developed using simple linear regression of 72 samples per genotype.

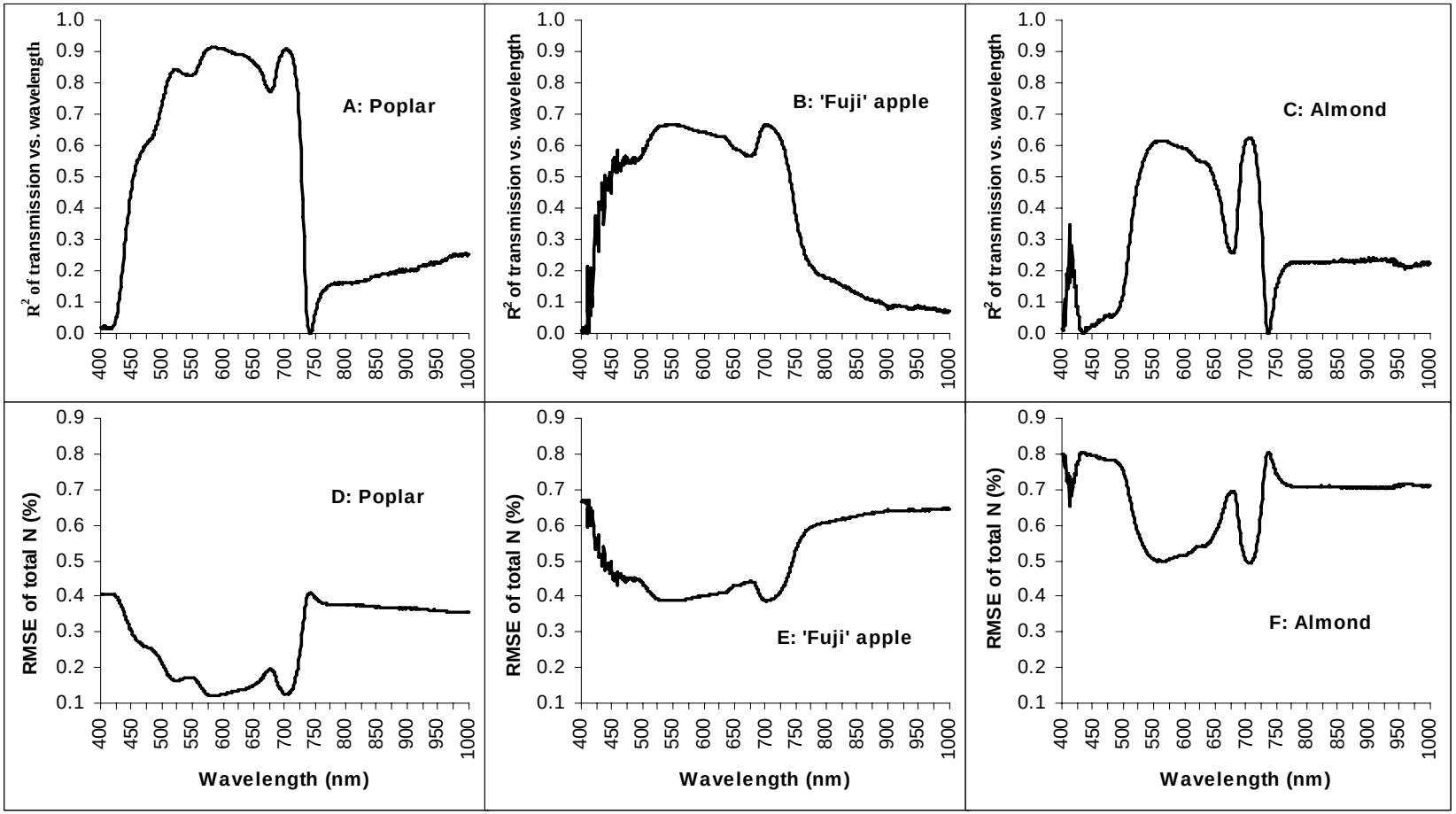


Figure 6.7 Curves of coefficient of determinations ($R^2$) and root mean square errors (RMSE) for the relationships between transmission values and nitrogen (N) concentration at 1 nm intervals from 300nm to 1100 nm in leaves of poplar (A, D), apple (B, E) and almond (C, F). Apple = *Malus domestica* 'Fuji'; Poplar = *Populus trichocarpa* x *P. deltoids*; Almond = *Prunus dulcis* 'Nonpareil'. Curves developed using simple linear regression of 72 samples per genotype.

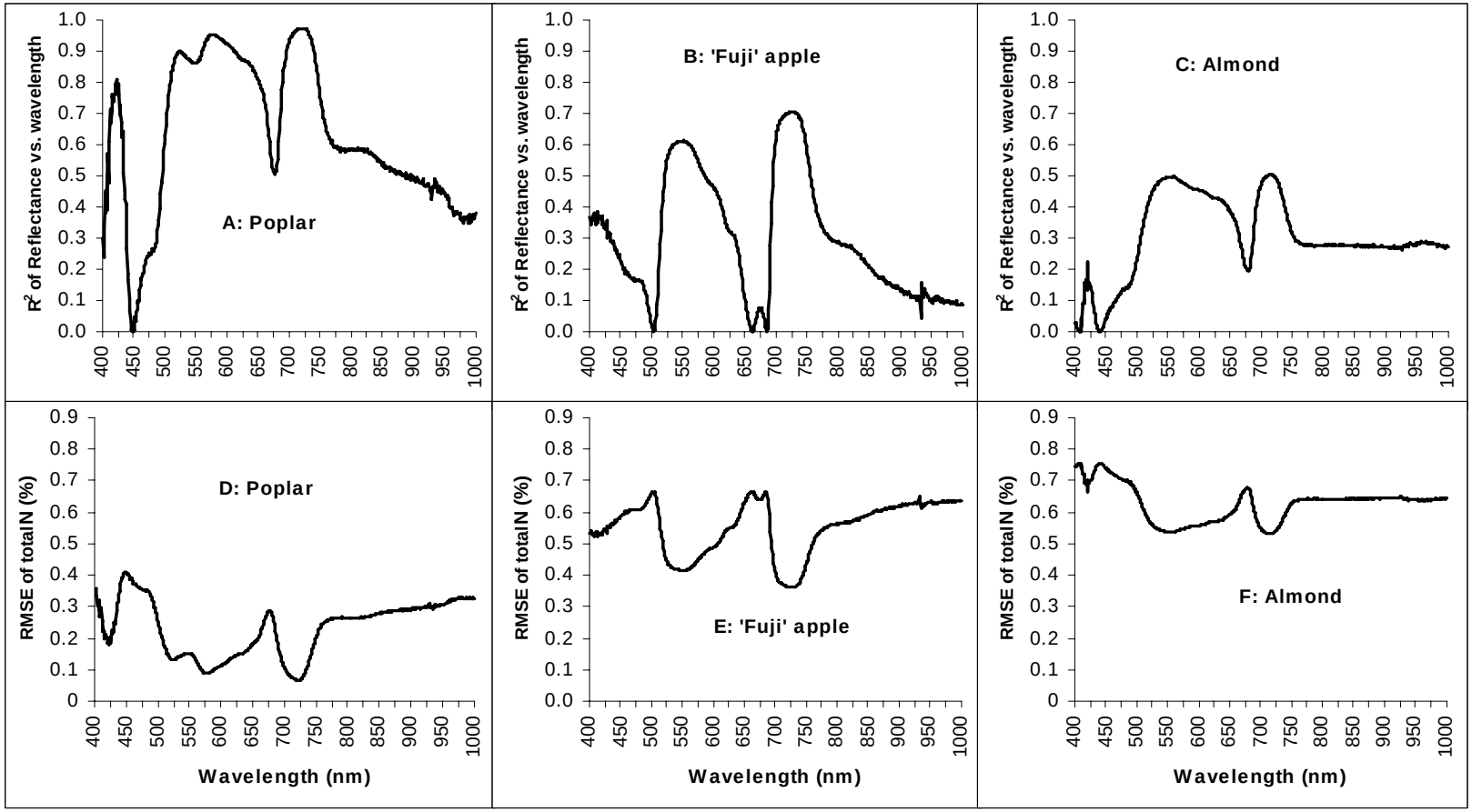


Figure 6.8 Curves of coefficient of determinations ($R^2$) and root mean square errors (RMSE) for the relationships between reflectance values and nitrogen (N) concentrations at 1 nm intervals from 300nm to 1100 nm in leaves of poplar (A, D), apple (B, E) and almond (C, F). Apple = *Malus domestica* 'Fuji'; Poplar = *Populus trichocarpa* x *P. deltoids*; Almond = *Prunus dulcis* 'Nonpareil'. Curves developed using simple linear regression of 72 samples per genotype.

# CHAPTER 7

# DISSERTATION SUMMARY

The goal of this research was to improve understanding of factors influencing the interactions that occur between leaves and spectral wavelengths and how these factors affect the accuracy of nondestructive, leaf-level chlorophyll (Chl) and nitrogen (N) assessment. Leaves with different leaf Chl and N concentrations from several different plant species and cultivars (e.g. almond, poplar, apple, purple leaf plum, and purple leaf flowering cherry) were analyzed using spectroradiometor, a scanning monochromator, hand-held meters, and chemical methods to (1) determine the best methods for selecting and using optimum wavelength (OW) to develop indices for Chl ($OW_{Chl}$) and N ($OW_N$) assessment ; (2) establish how methods for developing indices influence the accuracy of Chl assessment; (3) characterize how plant genotype and variation in leaf properties (texture, water status, and pigments) influence Chl assessment; (4) identify how parameters in hand-held meters used to assess Chl and N influence meter accuracy; (5) determine parameters that can be used by hand-held meter to increase accuracy of nondestructive Chl and N assessment; and (6) develop a hand-held meter with higher accuracy and sensitivity for nondestructive Chl and N assessment than available commercial hand-held meters.

We found that the 1$^{st}$ derivative method can be used to estimate $OW_{Chl}$ in the red edge region (700-730 nm) and the reflectance sensitivity analysis can be used to estimate the $OW_{Chl}$ in both the red edge (700-730 nm) and visible (540-580 nm) regions. However, neither the 1$^{st}$ derivative method nor the reflectance sensitivity analysis alone could accurately identify the actual $OW_{Chl}$. The reflectance sensitivity analysis is more

accurate and meaningful when used for $OW_{Chl}$ selection than the 1st derivative method, because methods used for reflectance sensitivity analysis ensures that the spectral differences are caused by the differences in Chl concentration, while the 1st derivative method does not distinguish whether spectral differences are the result of differences in Chl concentration or caused by other factors. Simple linear regression ($R^2$ and RSME) is a useful method for identifying OW used for Chl and N nondestructive assessment. Higher $R^2$ values are usually associated with lower RMSE and higher reflectance sensitivity. We believe that using $R^2$ in combination with reflectance sensitivity analysis is the most reliable method for determining OW for Chl and N assessment in plant leaves.

Using this reliable method, we determined that there are two ranges of OW having largest $R^2$ and highest sensitivity for Chl and N assessment, one in visible region (550 - 580 nm) and the other in the red edge region (700 - 730 nm). The variation in OW among species and Chl types (e.g. Chl a, Chl b, total Chl) is enough to impair the accuracy of an index and utility across a wide range of species. However, we found that the OW in the red edge region can be used as the "common" OW to accurately measure Chl and N among all species tested; whereas the OW in the visible region can be only used as the "common" OW among a wide range of anthocyanins-free species. The $OW_{Chl}$ from both the visible and red edge regions were sensitive over a wide-range of Chl concentrations (160 to 1188μmol.$m^{-2}$) in the species tested. Our research results indicate that differences in visible and red edge regions caused by variation of leaf texture or other optical properties can be reduced or eliminated by referencing a Chl-related $OW_{Chl}$ in the visible or red edge region to a reference wavelength (RW) either in blue (430 -490 nm) or NIR (750 -1100 nm) as simple ratio ($R_{RW}/R_{OW}$), normalized reflectance difference ($R_{RW}$ –

$R_{OW}$)/( $R_{RW}$+$R_{OW}$) or any other indices. The use of more than one wavelength is particularly important if the accuracy of the estimate obtained using either the Chl- or N-sensitive wavelength alone is small (e.g. $R^2$<0.8000 for Chl or $R^2$<0.6000 for N). The effect of leaf water status (e.g. dehydration) on spectral reflectance can be reduced by referencing a Chl-related $OW_{Chl}$ either from visible or red edge region to a water sensitive wavelength between 1420 - 1510 nm.

The accuracy of commercial hand-held meters is affected by not using OW for indices calculations and lack of uniformity in sampling distance and light intensity. We found that using transmission or reflectance from indices that use OW in either the visible or in red edge region is more accurate for Chl and N assessment than using the wavelengths employed by the commercial hand-held meters tested. Using the indices and OW determined from our research, we developed three meter prototypes 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 commercial hand-held meters for Chl assessment and than the CM-1000 for N assessment across all species we tested. These results verified our conclusion that more accurate hand-held meters for Chl and N assessment can be developed by (1) improving sampling distance precision, (2) increasing uniformity of target sample illumination, and (3) using an Chl- and N-sensitive OW from either the green or red edge region of the spectrum in combination with a Chl- and N-insensitive NIR wavelength in algorithms for predicting Chl and N concentrations.

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# APPENDICES

# APPENDIX A

# DEVELOPMENT OF A TRANSMMISION HAND-HELD METER FOR ASSESSING CHLOROPHYLL AND NITROGEN IN FRESH LEAVES

## A.1 Abstract

Three meter prototypes were developed using internal light-emitting diodes (LEDs) and constant sampling distance, and based on the OW and indices we identified in this study: (1) Prototype-I measures transmission of two Chl-related wavelengths (560 nm in green and 700 nm in red edge) and output a relative index value based on single wavelength index; (2) Prototype-II measures transmission of two Chl-related wavelengths (560 nm and 700 nm), one texture related wavelength in the NIR (940 nm) and one water-related wavelength in the MIR (1450 nm) and output both a relative index value and Chl and N concentrations based on indices of simple ratio and cultivar calibration; (3) Prototype-III was similar to Prototype-II and also includes a GPS receiver and an attached HP iPAQ PDA-5555 user interface. Prototype-III has more functions and can directly output relative index value, and Chl and N concentrations based on calibration and save the geographical coordinates for developing field maps of Chl and N status.

## A.2 Introduction

Efficient N management in crop production requires frequent assessment of plant N-status; however, the standard methods for N measurement are destructive and time consuming (Handson and Shelley 1993). Recently, nondestructive hand-held meters based the principles of light transmission (e.g. SPAD-502, Minolta Corp., Japan; CCM-200 Chl

Content Meter, Opti-Science, Inc., Tyngsboro, MA) or reflectance (CM 1000 Chl Meter, Spectrum Technologies, Inc., Plainfield, IL) have been developed and are used for conveniently assessing plant leaf Chl and N status in fresh leaves of a wide variety of plant genotypes, including annual, perennial and woody plants (Bullock and Anderson 1998, Costa et al. 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 these hand-held meters, however, are low and influenced by many factors including plant genotype (Bullock and Anderson 1998, Peng et al. 1993, Schepers et al. 1992, Sunderman and Lamm 1991, Takebe and Yoneyama, 1989. ), and leaf characteristics 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), water status (Martinez and Guiamet 2004), and meter design parameters. Our prior research (Chapters 3, Chapter 4, Chapter 5 and Chapter 6) have proven Chl-related optimal wavelengths (OW), constant sampling distance and light source are very important parameters for increasing meter accuracy. We found that the indices developed using the Chl-sensitive OW from the red edge (700- 730 nm) or visible (550 – 580 nm) regions in combination with reference wavelength (RW) from the near-infrared (NIR, 750 – 1100 nm) region that is sensitive to leaf structure but insensitive to Chl as the form of $R_{RW}/R_{OW}$ and $(R_{RW}-R_{OW})/(R_{RW}+R_{OW})$ could be used to accurately assess Chl and N in fresh leaves among species. To further increase indices accuracy, we also found that inclusion of a wavelength from middle infrared (MIR, 1420 - 1510 nm) can reduce the effect of leaf water status on indices accuracy (Chapter 5). We believe a new meter that uses superior indices in combination with constant light source and sampling distance design

could be developed more accurately than the commercial hand-held meters for Chl and N assessment. The objectives of this study were to (1) develop more accurate meters for assessing Chl and N using than commercial hand-held meters, (2) develop a method to directly generate output from a meter as Chl and N concentrations instead of output in the form of a relative index value.

## A.3 Materials and methods

### *A.3.1 Plant materials*

Apple (*Malus domestica* Borkh'Gala' and 'Fuji') trees 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). 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 create different Chl and N contents in the leaves.

### *A.3.2 Chlorophyll and N analysis and cultivar calibration*

In August, 12 fresh leaves from each cultivar in each N fertigation treatment were removed from trees and measured with the commercial hand-held meters and the Prototype-II and prototype-III. After measurement with hand-held meters, the Chl and N concentrations of leaves were determined using standard chemical methods. Discs (1 $cm^2$) were excised from each sample leaf with cork borer, cut into small pieces, then 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). N concentration of the remaining portion of each leaf was determined by the Kjeldahl procedure (Horneck et al., 1989). The meter index output results and the Chl and N chemical results were put into the software for cultivar calibration before used in the software for conversation from meter reading into Chl and N concentration (Figure A.8).

### *A.3.3 Development of meter prototypes*

Three meter prototypes were developed using internal light-emitting diodes (LEDs) and constant sampling distance in this study (Figure A.1): (1) Prototype-I measures transmission of two Chl-related wavelengths (560 nm in green and 700 nm in red edge) and calculates output based on single wavelength index; (2) Prototype-II measures transmission of two Chl-related wavelengths (560 nm and 700 nm), one texture related wavelength in the NIR (940 nm) and one water-related wavelength in the MIR (1450 nm) and calculates output based on a simple ratio (SR) index ($T_{940}/T_{700}$ or $T_{940}/T_{560}$); (3) Prototype-III was similar to Prototype-II and also includes a GPS receiver and an attached HP iPAQ PDA-5555 user interface. The output from Prototype-I is in the form of a relative index value. Prototype-II contains a small database allowing for output in the form of both a relative index and calculated Chl and N concentrations. Prototype-III has more functions and output types with more user information (e.g. meter raw index, Chl and N concentrations, and geographical positioning information for the sample tested). Using Chl and N concentration output and the GPS information (meters in the UTM coordinate system), the field maps of Chl and N concentration were developed

using the commercial software Surfer (Version 8.0, RockWare, Inc., Golden, Colorado). All three meter prototypes were developed in cooperation with Dr. Thomas K Plant, Guy Barnes and Dan Melende in the School of Electrical Engineering and Computer Science at Oregon State University.

### *A.3.4 Software development*

Using Microsoft Visual Basic 6.0 and Microsoft Visual Studio.Net 2003 (Microsoft Corp., Redmond, WA), two custom software applications were developed. One application was developed to convert the relative index output from the meter into Chl and N concentrations using a PC, the other application was developed for Prototype-III to perform similar conversions using a HP iPAQ PDA-5555 user interface and save GPS information.

## A.4 Results

### *A.4.1 Meter functions*

A.4.1.1 Prototype-I

A Prototype-I meter was developed with output in the form of a relative index value, similar to commercial meters (SPAD-502, CCM-200 and CM-1000). In order to convert the index value into Chl and N concentrations, the output of Prototype-I has to be uploaded to a PC and converted into Chl and N concentrations with the software we specifically developed for use with the meter. The Prototype-I was built to store results from up to 500 samples.

A.4.1.2 Prototype-II

A Prototype-II meter was developed with more functions than Prototype-I and a storage capability for results from up to 1000 samples. A small database was included in the Prototype-II that contains regression calibration parameters for different plant cultivars. These calibration parameters are used for converting relative index values into Chl and N concentrations directly in the hand-held unit. Prototype-II user interface displays output as Chl and N concentrations or relative index values. The schematics of the main functions developed for Prototype-II are shown in Figure A.2. After the power button is pushed to the *ON* position the Prototype-II will first display *PROTOTYPE-II METER READY*, followed by a menu that allows the user to select the operating *MODE*. There are 6 main modes (*MEASURE*, *VIEW*, *DELETE*, *PLANT*, *CLEAR*, and *UPLOAD*) marked as numbers 1 - 6.

The *MEASURE* mode enables the user to calibrate the meter by closing the sampling head without any test sample. After calibration, the meter is ready to be used for measuring samples. In the *MEASURE* mode, Prototype-II will display the value for the test sample as the raw sample reading of the different wavelengths. The *VIEW* mode displays the data that has been stored in the meter. The user can view the next or previous stored data by pressing *A.NEXT* or *C.PREV*. The *DELETE* mode enables user to delete the stored data one measurement at a time from the most recent data stored. The *PLANT* mode enables the user to select a plant group, species or specific cultivar, then use the regression calibration parameters stored in the meter for the specified cultivar to convert index values to Chl and N concentrations. The *CLEAR* mode clears all data stored in the meter and releases the storage space. The *UPLOAD* mode enables the user to upload the data to a PC for future analysis.

A.4.1.3 Prototype-III

A Prototype-III meter was developed with more functions than Prototype-II. It has a database for storing calibration information for specific cultivars and the ability to perform calibration automatically. After calibration, the relative index values generated by the meter can be directly converted into Chl and N concentrations based on cultivar calibrations. The Prototype-III is also equipped with a GPS receiver and has a HP iPQA PDA-5555 as the user interface. Synchronous output from the Prototype-III includes meter raw index values (PI (1), PI(2), PI(3) and PI(4)) generated by the detectors for different wavelength used in the meter, Chl (Chl in $\mu g.m^{-2}$) and N (NC in %) concentrations (Figure A.4II-III), GPS status (Figure A.3III-IV) and geographical positioning information (meters in the UTM coordinate system) (Table A). Using Chl and N concentration output and GPS information, it is possible to develop field maps of Chl (Figure A.5) and N (Figure A.6) status using commercial software surfer 8.0.

Although the meter Prototype-III can directly output Chl and N concentrations, users still do not know whether the N status in the plant is in the proper concentration range without comparing the leaf analysis standard. Therefore, it might be useful to incorporate current industry leaf analysis standards into future versions of the meter software allowing the user to immediately determine whether the plant N is in the optimum range. Currently with the output results of the meter Prototype-III we can develop Chl and N concentration maps using commercial software surfer 8.0; however, this may not be convenient for users. Moreover, the map created with Surfer 8.0 also does not show whether the plant N is in optimum range. Therefore, in the further development,

we will develop a function to use the current leaf analysis standard directly draw the field Chl and N concentration map in different color to indicate whether plant N or Chl is in proper or optimum range.

### A.4.2 Software

A Windows-based software application was developed to convert relative index values generated by all three prototype meters into estimates of Chl and N concentrations in fresh leaves. The software has a database to store the calibrations for different species and cultivars. The calibrations are automatically performed by the software after user input of meter index values and the results from chemical analyses of leaves for Chl and N concentrations. After calibration for specific species or cultivars, the software will directly convert index values into Chl and N concentrations (Figure A.7, Figure A.8, and Figure A.9). This software can also convert index values from the commercial meters (SPDA-502, CCM-200 and CM-1000) into Chl and N concentrations.

## A.5 Conclusions and further research

Our results have shown that it is possible to develop meters with better accuracy for assessing Chl and N in plant leaves than currently available meters (Chapter 6). Software developed can be used on PCs or directly on hand-held units to improve usability by allowing users to record or convert relative index values generated by meters to Chl and N concentration units. The software allows user to calibrate information for species and cultivars for converting meter index output directly into of Chl and N contents. Capabilities of hand-held meters were extended by the addition of GPS information in

conjunction with leaf Chl and N measurement results allowing users to generate field maps of Chl and N status. Future meter capabilities could include more species and cultivar calibrations, tissue optimums for specific crops for comparison to measured values, and PC based software specific for drawing Chl and N field maps using GPS data.

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Table A. Sample output generated by the Prototype-III meter for determining chlorophyll and N status of twelve 'Gala' apple leaves.

| SN[z] | PI (1)[y] | PI (2)[y] | PI (3)[y] | PI (4)[y] | Chl[x] ($\mu g.m^{-2}$) | N[x] (%) | Easting[w] | Northing[w] | Date[v] | Time[v] |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 54.28 | 76.76 | 76.78 | 61.96 | 1027.78 | 2.79 | 482901 | 4933286 | 8/4/2004 | 9:16:21am |
| 2 | 43.93 | 75.64 | 76.18 | 62.31 | 902.73 | 2.58 | 482904 | 4933282 | 8/4/2004 | 9:16:34 am |
| 3 | 53.75 | 74.31 | 61.06 | 64.46 | 1021.39 | 2.78 | 482903 | 4933284 | 8/4/2004 | 9:17:32 am |
| 4 | 9.41 | 70.08 | 59.39 | 56.6 | 485.55 | 1.88 | 482904 | 4933279 | 8/4/2004 | 9:18:14 am |
| 5 | 44.99 | 73.87 | 73.21 | 58.93 | 915.52 | 2.6 | 482903 | 4933281 | 8/4/2004 | 9:18:28 am |
| 6 | 22.08 | 72.64 | 64.63 | 72.14 | 638.66 | 2.13 | 482902 | 4933280 | 8/4/2004 | 9:18:39 am |
| 7 | 21.02 | 71.08 | 67.01 | 63.91 | 625.87 | 2.11 | 482903 | 4933280 | 8/4/2004 | 9:18:52 am |
| 8 | 45.63 | 72.75 | 70.59 | 63.04 | 923.22 | 2.61 | 482901 | 4933280 | 8/4/2004 | 9:19:06 am |
| 9 | 21.66 | 70.98 | 67.96 | 63.04 | 633.57 | 2.13 | 482903 | 4933277 | 8/4/2004 | 9:19:26 am |
| 10 | 25.14 | 70.86 | 72.13 | 67.49 | 675.6 | 2.2 | 482899 | 4933276 | 8/4/2004 | 9:19:35 am |
| 11 | 46.25 | 72.75 | 71.06 | 63.38 | 930.8 | 2.63 | 482897 | 4933274 | 8/4/2004 | 9:19:44 am |
| 12 | 42.78 | 72.08 | 71.06 | 66.6 | 888.76 | 2.56 | 482894 | 4933272 | 8/4/2004 | 9:19:54 am |

[z]Sample number
[y]Meter index raw value estimated by different detectors via transmission. The output of meter raw value is in the form of PINGS index [(PI). PI(1), PI(2), PI(3) and PI(4)].
[x]Estimated leaf chlorophyll (Chl) and N concentration in leaves
[w]Position information used to located sample position (meters in the UTM coordinate system)
[v]Date and time are the current date and time when the sample measured


Figure A.1 Three meter prototypes

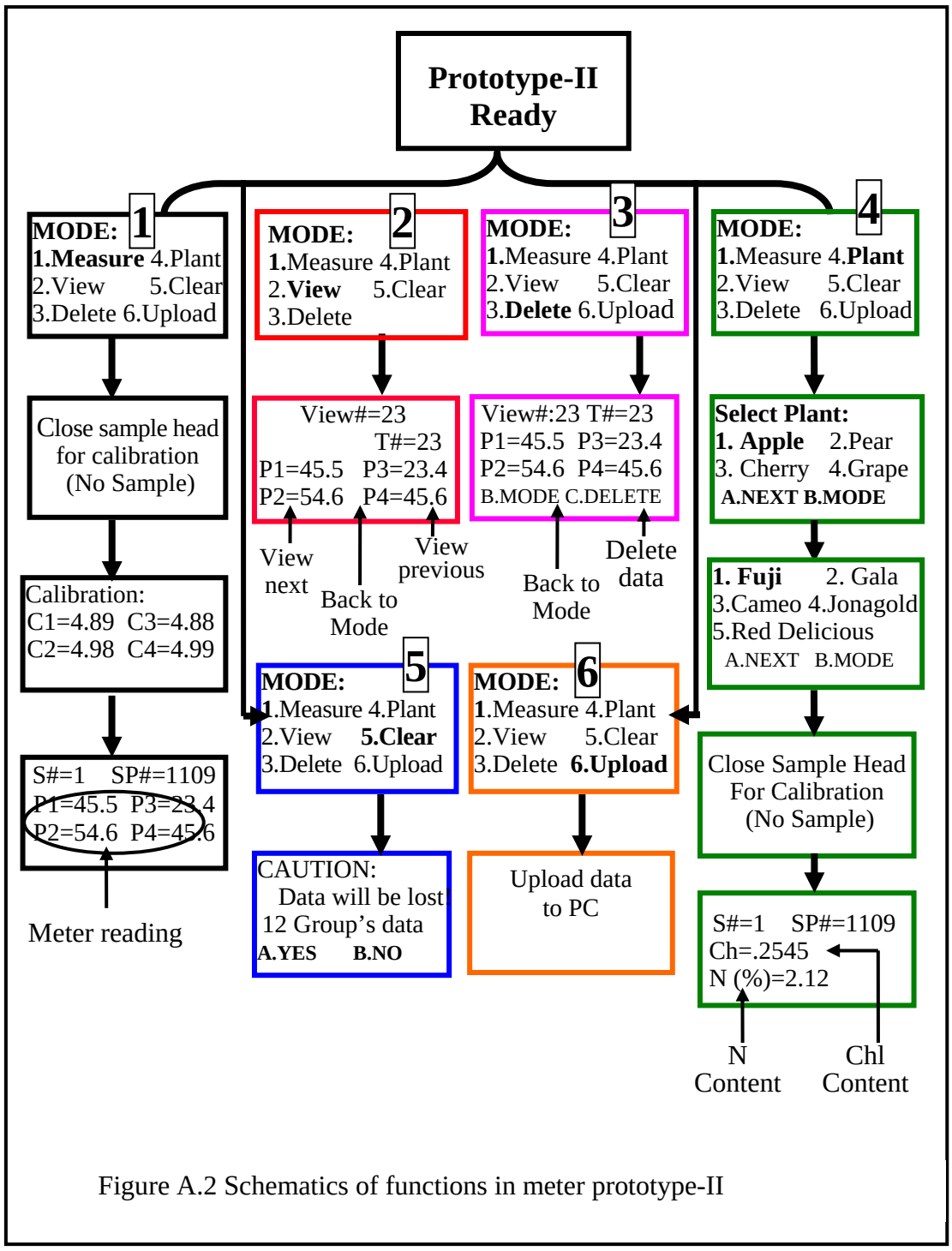



Figure A.2 Schematics of functions in meter prototype-II

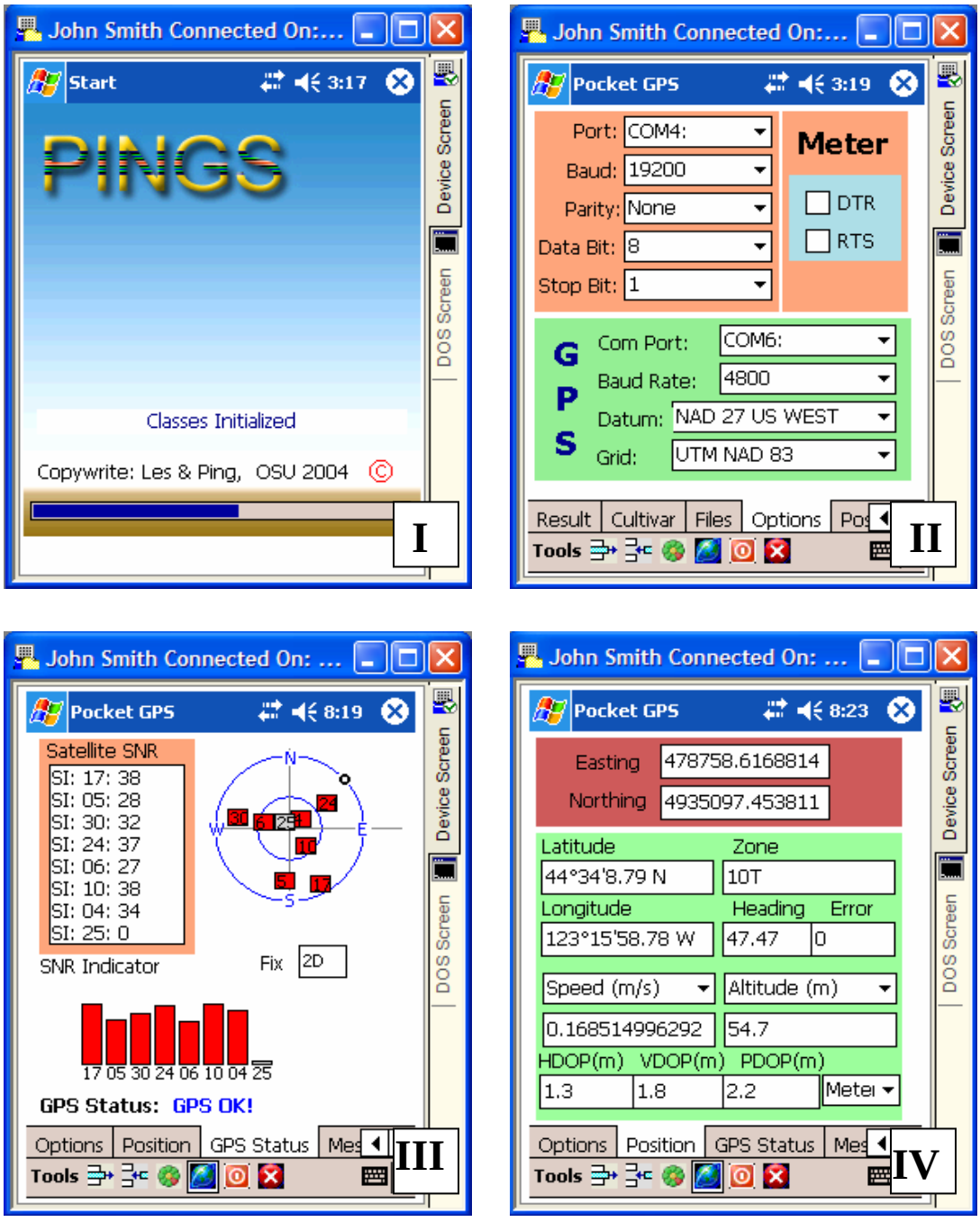
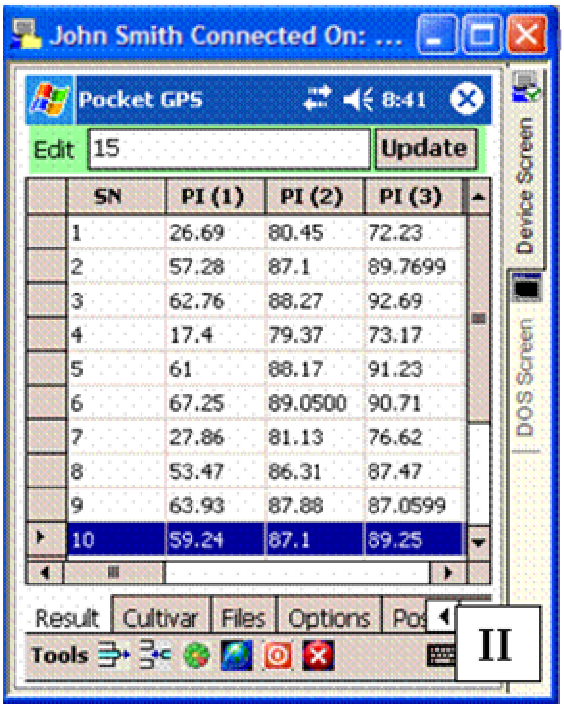



Figure A.3 Screen shots of meter and software functions of Prototype-III. I. Start-up screen; II. Options screen for selecting meter and GPS parameters; III. GPS Status screen; and IV. Position screen showing GPS information

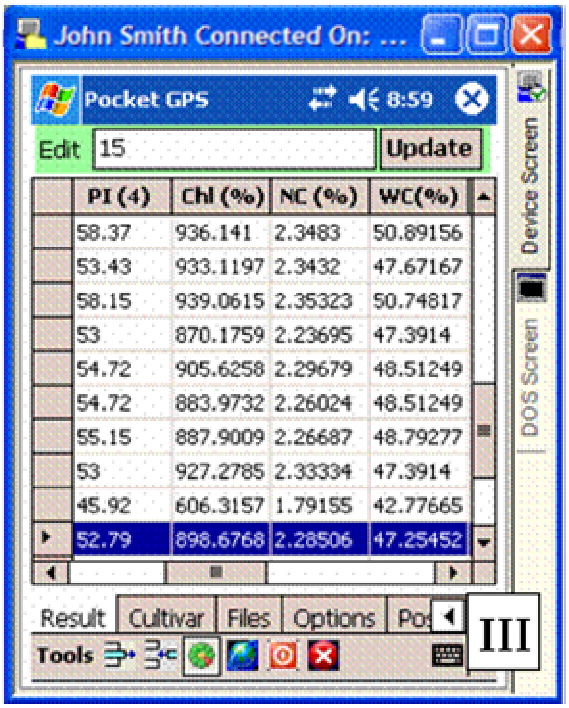




Figure A.4 Pot-in-pot 'Gala' apple trees grown in Lewis-Brown Horticulture Farm in Corvallis, Oregon (I) and screen shots (II and III) of Results screen from Prototype-III showing meter index values (PI(1), PI(2), PI(3) PI(4)), chlorophyll (Chl %) and N (NC%) concentrations and leaf water content (WC%).

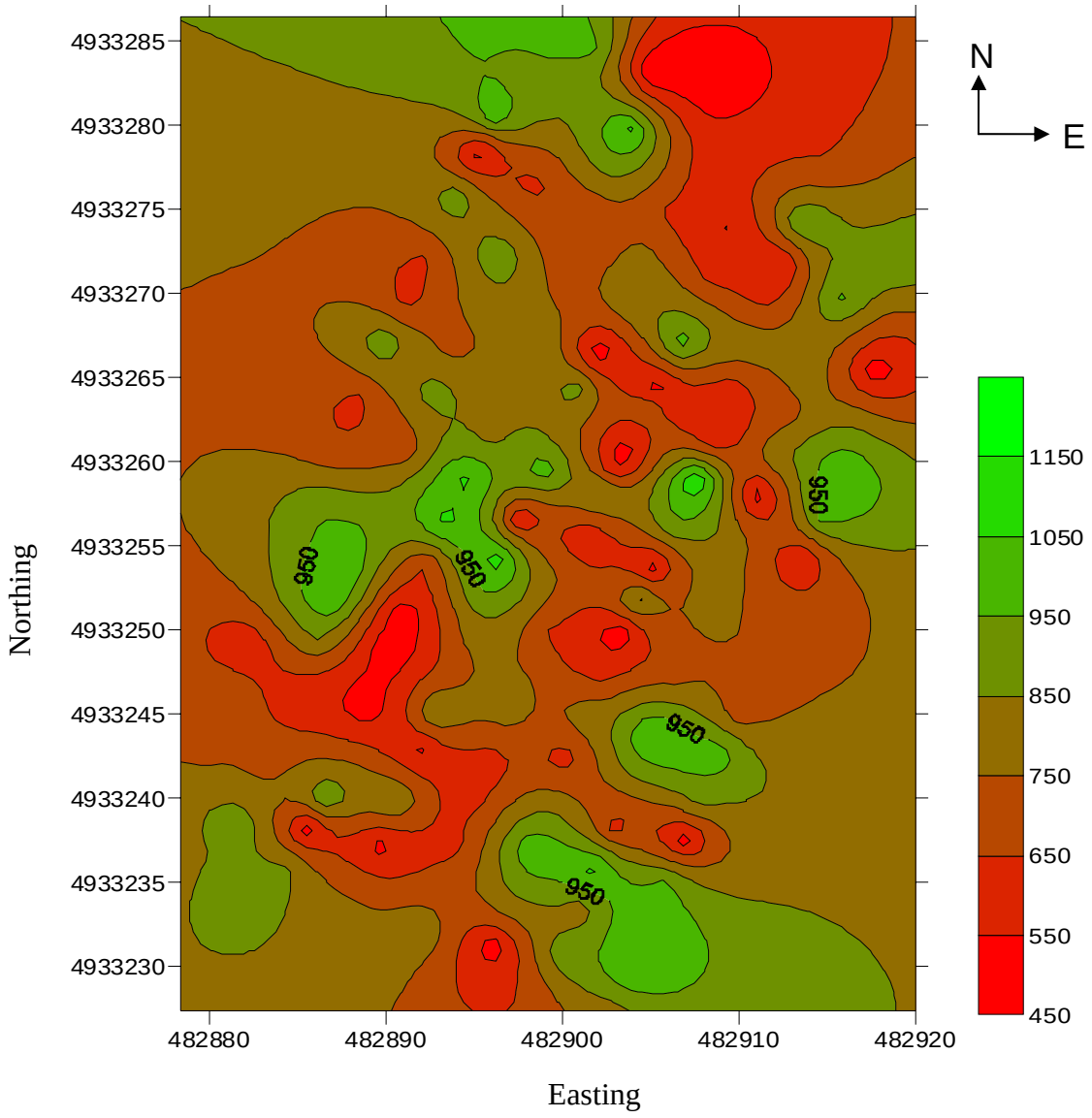



Figure A.5 Map of chlorophyll concentrations (µmol.m$^{-2}$) in leaves of pot-in-pot 'Gala' apple trees growing in Lewis-Brown Horticulture Farm in Corvallis, Oregon. Map developed based on the chlorophyll estimates and GPS information generated from Prototype-III by using Surfer (Version 8.0, RockWare, Inc., Golden, Colorado). Horizontal axis is the easting in meters used in UTM coordinate system; vertical axis is the northing in meters used in UTM coordinate system.

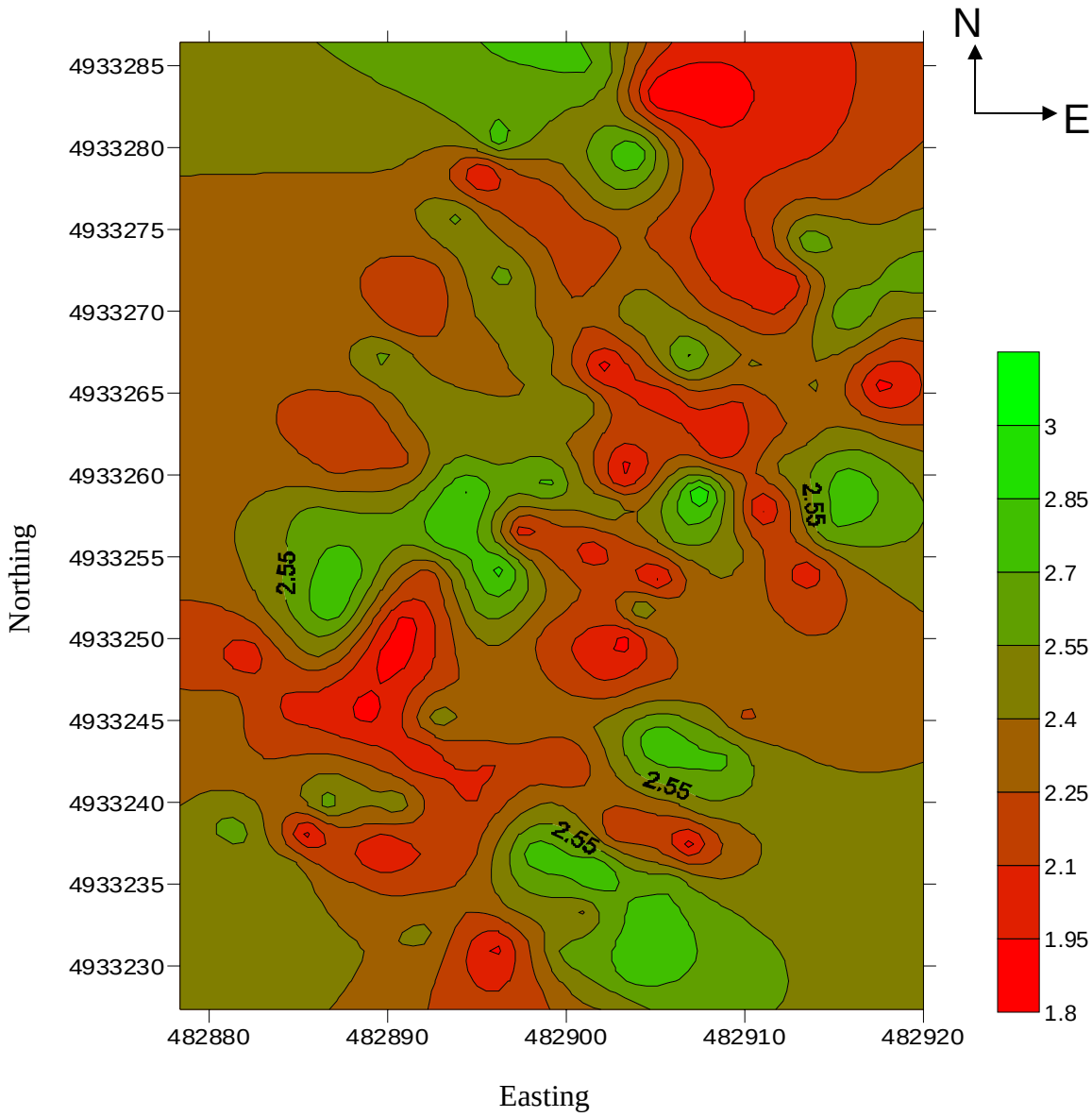
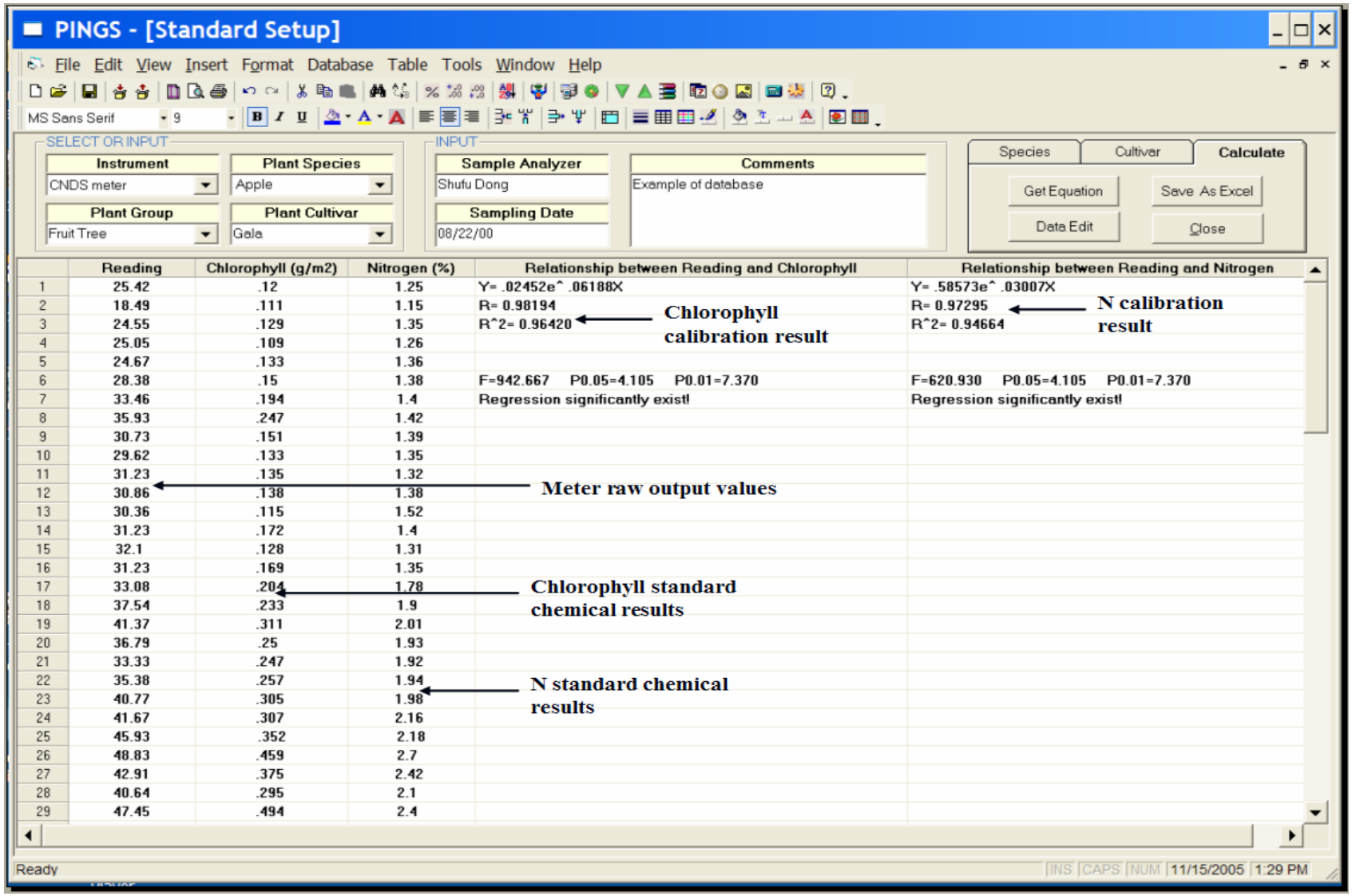



Figure A.6. Map of nitrogen (N) concentrations (%) in leaves of pot-in-pot ‘Gala' apple trees growing in Lewis-Brown Horticulture Farm in Corvallis, Oregon. Map developed based on the N estimates and GPS information generated from Prototype-III by using Surfer (Version 8.0, RockWare, Inc., Golden, Colorado). Horizontal axis is the easting in meters used in UTM coordinate system; vertical axis is the northing in meters used in UTM coordinate system.


Figure A.7 Screen shot of the PINGS software start-up screen used for developing calibration equations and converting index values from meters to chlorophyll and nitrogen concentrations




| | Reading | Chlorophyll (g/m2) | Nitrogen (%) | Relationship between Reading and Chlorophyll | Relationship between Reading and Nitrogen |
|---|---|---|---|---|---|
| 1 | 25.42 | .12 | 1.25 | Y= .02452e^ .06188X | Y= .58573e^ .03007X |
| 2 | 18.49 | .111 | 1.15 | R= 0.98194 | R= 0.97295 |
| 3 | 24.55 | .129 | 1.35 | R^2= 0.96420 | R^2= 0.94664 |
| 4 | 25.05 | .109 | 1.26 | | |
| 5 | 24.67 | .133 | 1.36 | | |
| 6 | 28.38 | .15 | 1.38 | F=942.667 P0.05=4.105 P0.01=7.370 | F=620.930 P0.05=4.105 P0.01=7.370 |
| 7 | 33.46 | .194 | 1.4 | Regression significantly exist! | Regression significantly exist! |
| 8 | 35.93 | .247 | 1.42 | | |
| 9 | 30.73 | .151 | 1.39 | | |
| 10 | 29.62 | .133 | 1.35 | | |
| 11 | 31.23 | .135 | 1.32 | | |
| 12 | 30.86 | .138 | 1.38 | | |
| 13 | 30.36 | .115 | 1.52 | | |
| 14 | 31.23 | .172 | 1.4 | | |
| 15 | 32.1 | .128 | 1.31 | | |
| 16 | 31.23 | .169 | 1.35 | | |
| 17 | 33.08 | .204 | 1.78 | | |
| 18 | 37.54 | .233 | 1.9 | | |
| 19 | 41.37 | .311 | 2.01 | | |
| 20 | 36.79 | .25 | 1.93 | | |
| 21 | 33.33 | .247 | 1.92 | | |
| 22 | 35.38 | .257 | 1.94 | | |
| 23 | 40.77 | .305 | 1.98 | | |
| 24 | 41.67 | .307 | 2.16 | | |
| 25 | 45.93 | .352 | 2.18 | | |
| 26 | 48.83 | .459 | 2.7 | | |
| 27 | 42.91 | .375 | 2.42 | | |
| 28 | 40.64 | .295 | 2.1 | | |
| 29 | 47.45 | .494 | 2.4 | | |

Figure A.8 Screen shot of the PING software Standard Setup screen showing calibration information of specific cultivars based on output from meter (Reading) and chlorophyll and nitrogen concentrations measured by standard chemical methods

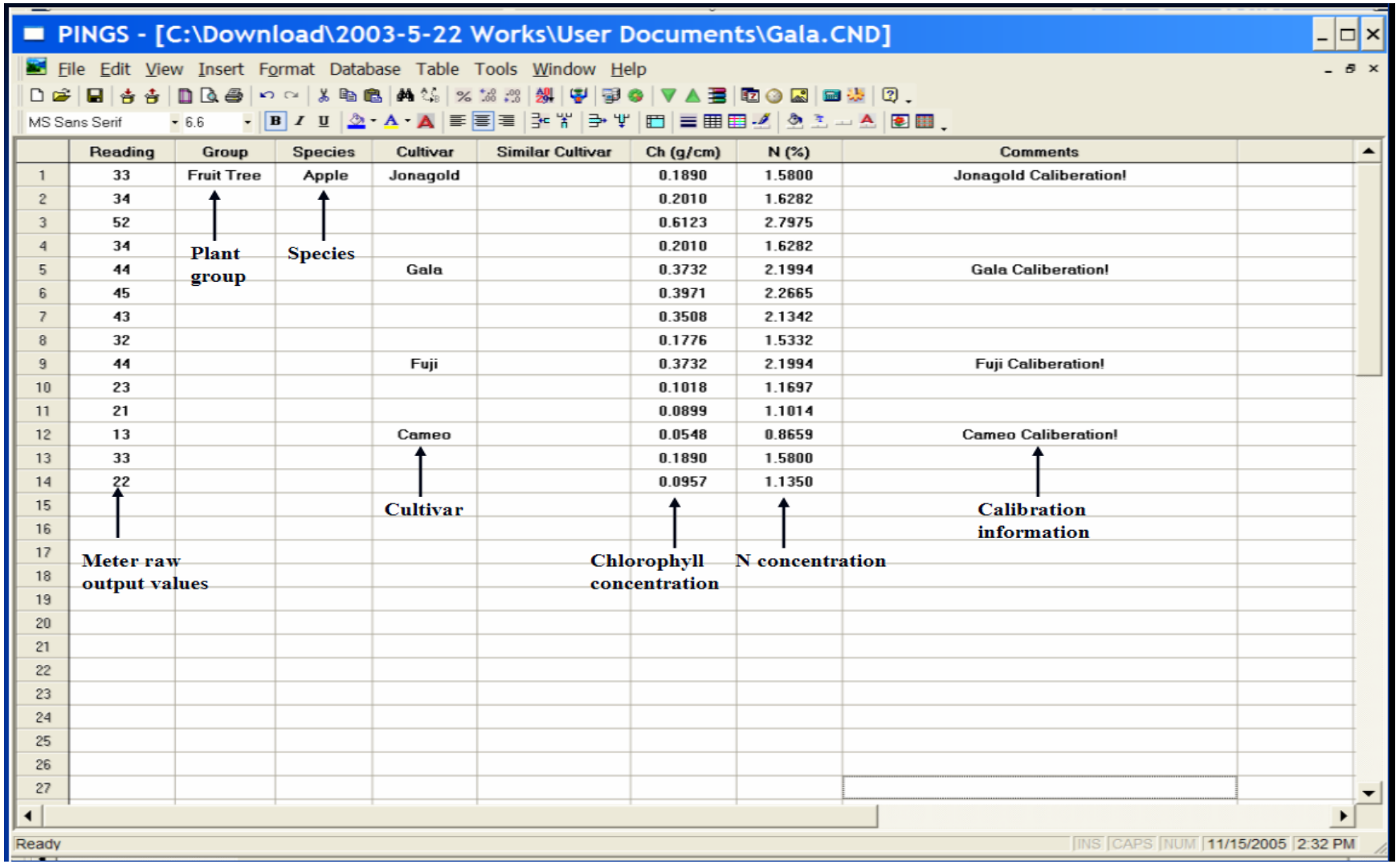



Figure A.9 Screen shot of the PING software Conversion screen showing conversion of meter output (Reading) into chlorophyll and N concentrations based the calibration for specific cultivars

## APPENDIX B.

CONCENTRATION OF TOTAL CHLOROPHYLL (CHL) AND NITROGEN (N) IN LEAVES OF DIFFERENT GENOTYPES TESTED IN THE STUDY

| Genotype[z] | Ranges of Chl concentration[y] ($\mu mol.m^{-2}$) | Ranges of N[x] concentration (%) |
|---|---|---|
| Almond | 173.32~710.45 | 1.0375~4.4906 |
| 'Cameo' apple | 276.34~935.65 | 1.5334~3.6533 |
| 'Gala' apple | 252.34~725.65 | 1.3455~3.1243 |
| 'Fuji' apple | 261.55~1188.72 | 1.4097~4.2557 |
| 'Jonagold' apple | 277.87~885.66 | 1.4563~3.2232 |
| Poplar | 160.45~659.54 | 0.8671~2.9959 |
| Purple leaf flowering cherry | 297.29~750.20 | 1.5434~2.8843 |
| Purple leaf plum | 165.18~505.50 | 1.0234~2.6554 |

[z]Almond = *Prunus dulcis* 'Nonpareil'; Cameo = *Malus domestica* 'Cameo' on M9 rootstock; Fuji = *Malus domestica* 'Fuji' on M9 rootstock; Gala = *Malus domestica* 'Gala' on M9 rootstock; Jonagold = *Malus domestica* 'Jonagold' on M9 rootstock; Poplar = *Populus trichocarpa* x *P. deltoids* 'UCC-1'; Purple leaf flowering cherry = *Prunus blireiana* **;** Purple leaf plum = *Prunus cerasifera* 'Newport'

[y]Ranges of chlorophyll (Chl) concentration in leaves of different genotypes from low to high

[x]Ranges of nitrogen (N) concentration in leaves of different genotypes from low to high