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Simple Linear Regression and Reflectance Sensitivity Analysis Used to Determine the Optimum Wavelengths for the Nondestructive Assessment of Chlorophyll in Fresh Leaves Using Spectral Reflectance

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Abstract. **The accuracy of nondestructive optical methods for chlorophyll (Chl) assessment based on leaf spectral characteristics depends on the wavelengths used for Chl assessment. Using spectroscopy, the optimum wavelengths (OW) for Chl assessment were determined by using 1-year-old almond (*Prunus dulcis*), poplar (*Populus trichocarpa* × *P. deltoides*), and apple (*Malus* ×*domestica*) trees grown at different rates of nitrogen fertilization to produce leaves with different Chl concentrations. Spectral reflectance of leaf discs was measured using a spectroradiometer (300 to 1100 nm at 1-nm intervals), and total Chl concentration in leaf discs was extracted and determined in 80% acetone. The OW for nondestructive Chl assessment by reflectance spectroscopy was estimated using 1) the coefficient of determination (r^2) from simple linear regression; 2) reflectance sensitivity analysis (a measure for changes of spectral reflectance on unit change in leaf Chl concentration); and 3) the first spectral derivative method. Our results indicated that the first derivative method can be used only to identify OW in the red edge region of the spectrum, whereas r^2 and reflectance sensitivity analysis can be used to identify the OW in both the red edge and green regions. Our results indicate that using simple linear r^2 in combination with reflectance sensitivity and/or the first derivative analyses is a reliable method for determining OW in plant leaves tested. Two optimum wavebands with larger r^2, smaller root mean square error, and higher reflectance sensitivity were found in red edge (700 to 730 nm) and green (550 to 580 nm) regions, respectively, which can be used as common OW for Chl reflectance assessment in poplar, apple, and almond leaves tested. Single-wavelength indices if developed with OW were even more accurate than those more wavelength indices that developed without using OW. The accuracy of indices can be further improved if indices developed by using one OW and one Chl-insensitive wavelength from near infrared (NIR) (750 to 1100 nm) in the form of R_{NIR}/R_{OW} or $(R_{NIR} - R_{OW})/(R_{NIR} + R_{OW})$.**

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). As much as 75% of the total nitrogen (N) in a plant is required for normal chloroplast formation (Kutik et al., 1995) and synthesis of components of the photosynthetic apparatus, including thylakoid membranes and photosynthetic enzymes (Evans, 1989); therefore, Chl concentration gives an indirect estimation of plant N status and photosynthetic potential (Filella et al., 1995; Moran et al., 2000). Leaf Chl concentration is often closely related to plant stress and can be used as an indicator of plant stress (Carter, 1993, 1994; Carter and Knapp, 2001; Peñuelas and Filella, 1998).

Traditionally, leaf Chl was extracted with organic solvents and measured using a spectrophotometer (Lichtenthaler, 1987; Lichtenthaler and Wellburn, 1983). Recently, alternative

Table 1. Effect of nitrogen concentration in fertigation solution on chlorophyll concentration in apple, poplar, and almond leaves.

Nitrogen	Total chlorophyll concn (μmol·m^{-2})		
concn (mM)	Fuji apple	UCC-1 poplar	Nonpareil almond
0.0	261.52 a[z]	160.82 a	173.33 a
2.5	438.51 b	238.74 b	298.41 b
5.0	608.53 c	383.64 c	418.20 c
7.5	772.10 d	436.71 d	478.50 d
10.0	947.44 e	529.20 e	584.46 e
20.0	1,188.33 f	659.24 f	710.44 f

[z]Means of chlorophyll concentration within a column not followed by the same letter are significantly different based on Tukey pairwise comparison (n = 12).

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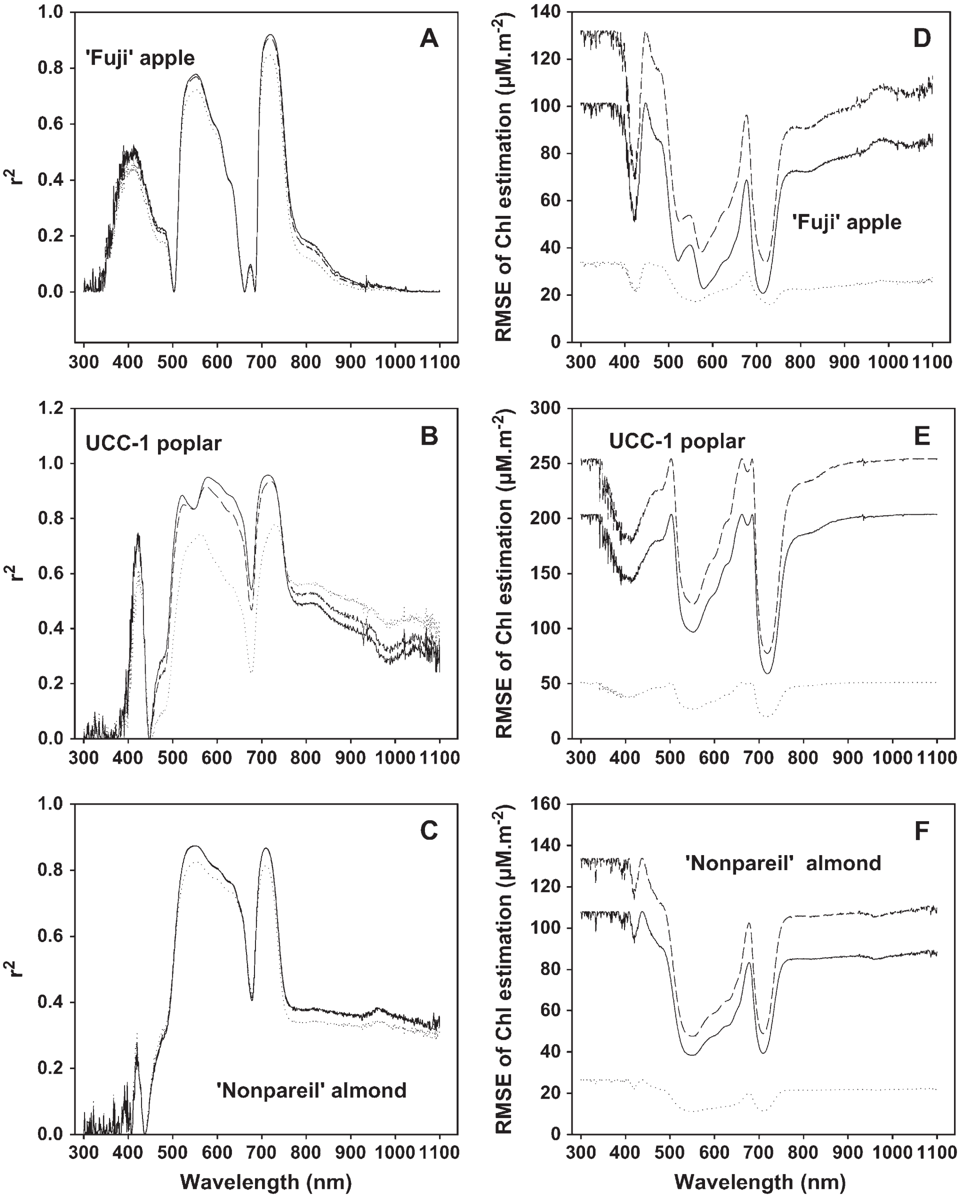

Fig. 1. Coefficients of determination (r^2) and root mean square errors (RMSE) for the relationships between chlorophyll concentrations (Chl a, Chl b, and Chl a + b) and reflectance values from 300 to 1100 nm in leaves (n = 72) of apple (**A, D**), poplar (**B, E**), and almond (**C, F**), respectively

nondestructive optical methods based on leaf light absorbance and/or reflectance have been developed (Adams et al., 1999; Curran et al., 1990; Datt, 1999; Gamon and Surfus, 1999). These optical methods require no chemical analysis, are nondestructive, simple to use, fast, inexpensive, and can be used in field conditions (Buschmann and Nagel, 1993; Gitelson and Merzlyak, 1994; Gitelson et al., 1996a, 1996b). 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) (Carter, 1994, 1998; Jacquemoud and Baret, 1990; Moran and Moran, 1998; Thomas and Gausman, 1977); 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 (Adams et al., 1999; Blackburn, 1998; Curran et al., 1990; Datt, 1998, 1999; Gamon and Surfus, 1999; Gitelson and Merzlyak, 1994, 1996; Gitelson et al., 1996a, 1996b); however, the applicability of the proposed indices was 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).

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

		Ultraviolet region		Green region		Red edge region	
Species	Chlorophyll	λ (nm)	r^2	λ (nm)	r^2	λ (nm)	r^2
Fuji apple	Chl a	412	0.526 aABC*[z]	552	0.779 bAB**	720	0.920 cBCD***
	Chl b	390	0.476 aAB*	550	0.723 bA**	717	0.847 bABC***
	Chl a + b	410	0.524 aABC*	552	0.770 bAB**	720	0.907 cBC***
UCC-1 poplar	Chl a	422	0.747 aC**	581	0.950 bD***	715	0.958 bD***
	Chl b	423	0.613 aB**	563	0.744 bAB**	730	0.780 bA**
	Chl a + b	422	0.733 aC**	575	0.917 bCD***	720	0.935 bCD***
Nonpareil almond	Chl a	420	0.263 aA NS	549	0.874 bBC***	710	0.868 bABC***
	Chl b	420	0.312 aA*	558	0.826 bABC***	710	0.814 bAB***
	Chl a + b	420	0.276 aA NS	550	0.874 bBC***	710	0.867 bABC***

[z]NS, *, **, ***Nonsignificant or significant at $P < 0.05$, 0.01, or 0.001, respectively; 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).

There are many reasons why reported indices or algorithms are not applicable for Chl assessment across genotypes or different studies. However, one of the main reasons is that the optimum wavelength (OW) for measuring Chl used in one study differed from it used in other studies. Differences in OW 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 for developing Chl-related indices is the use of the first derivative of reflectance spectra (Curran et al., 1990; Gitelson et al., 1996a, 2003; Kochubey and Kazantsev, 2007; Richardson et al., 2002). First derivatives can be used to resolve or enhance smaller peaks that are incompletely resolved from larger peaks as a result of either the background or noise (Curran et al., 1990; Moran et al., 2000) and have been successfully used for identifying the red edge waveband for Chl assessment (Curran et al., 1990; Gitelson et al., 1996a, 2003; Kochubey and Kazantsev, 2007). However, derivative changes the original peak form and may eliminate some important peaks.

Different 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 accuracy (goodness of fit) of regression response at specific wavelength to Chl concentration. Theoretically, the OW 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 for Chl-related waveband identification (Carter and Spiering, 2002; Gitelson et al., 2003; Read et al., 2002). Reflectance sensitivity has been used in some studies to identify stress-sensitive Chl-related wavelengths (Carter, 1993, 1994; Moran et al., 2000) but not used in combination with r^2 to determine OW. Our objectives were to 1) evaluate whether simple linear regression in combination with reflectance sensitivity analysis can be used to determine OW for Chl assessment using reflectance; and 2) evaluate the importance of using OW in the development of Chl-related reflectance indices.

Materials and Methods

Plant materials. In 1999, 2000, and 2002, 1-year-old trees of 'Nonpareil' almond, UCC-1 poplar (Union Camp, Princeton, NJ), and 'Fuji' apple on M.26 rootstocks were grown in 7.2-L pots containing 1 peatmoss:2 pumice:1 sandy loam soil (v:v) in a lath house in Corvallis, OR (lat. 44°30′ N, long. 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 20N–4.4P–16.6K water-soluble fertilizer with micronutrients (Plantex® 20-10-20; Plantex Corp., Brampton, Ontario, Canada). When new shoots were ≈15 cm long, plants were moved to full

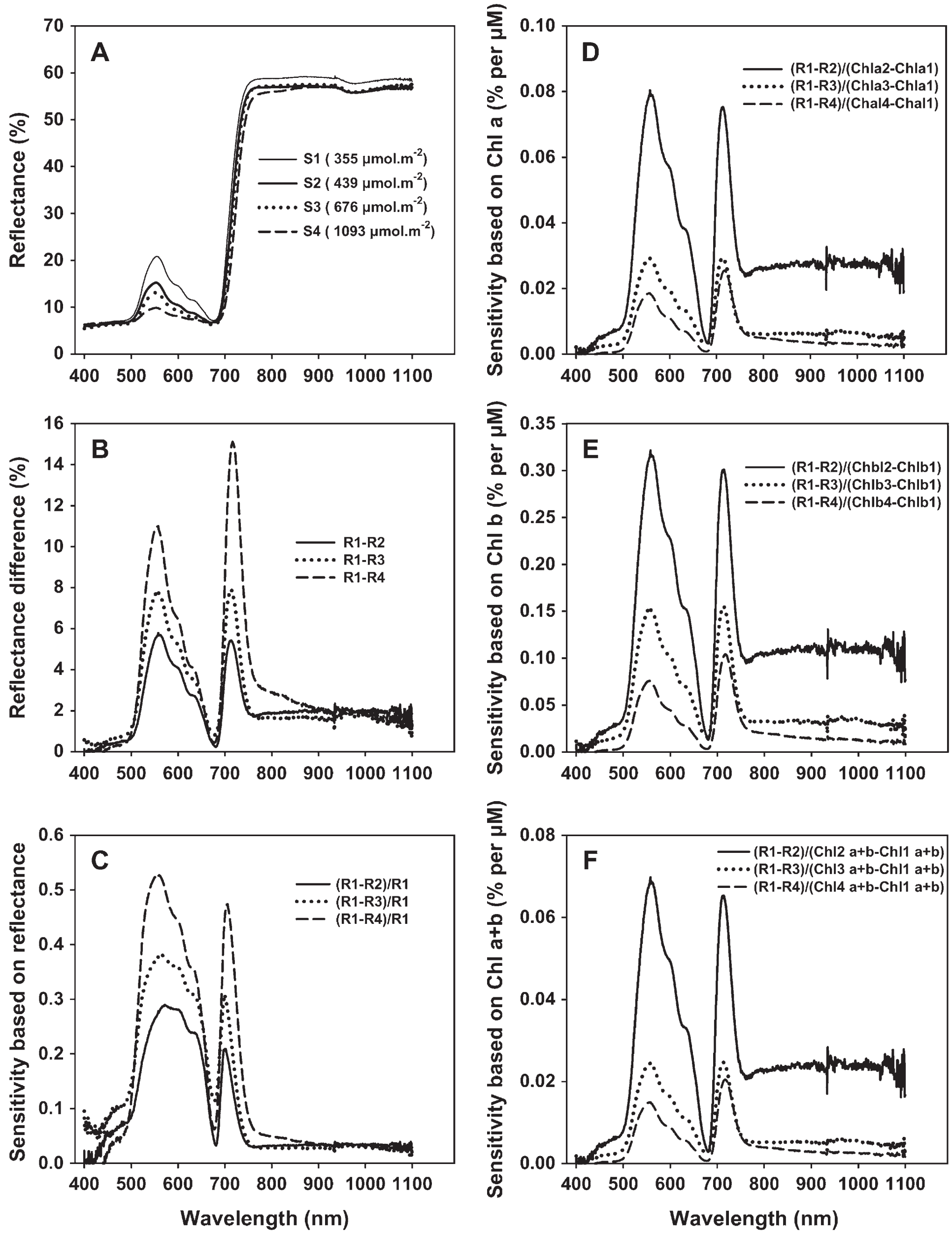

Fig. 2. Spectra of reflectance (**A**), reflectance difference (**B**), and reflectance sensitivity (**C–F**) of four 'Fuji' apple leaves (S1–S4) with different chlorophyll concentrations (Chl1–Chl4). The reflectance difference (**B**) was calculated by subtracting the reflectance of S2, S3, and S4 (R2, R3, and R4) from the reflectance of S1 (R1). The sensitivity curves based on reflectance (**C**) were calculated by dividing the reflectance difference by R1 (no unit). The sensitivity based on Chl a, Chl b, and Chl a + b (**D–F**) was calculated by dividing the reflectance difference by the difference in concentrations of Chl a, Chl b, and Chl a + b among leaf samples S2, S3, S4, and S1.

sunlight and fertilized weekly with Plantex® 20-10-20 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.

Spectral reflectance and chlorophyll determination. In August and September, 12 fresh leaves from each species (genotype) in each N fertigation treatment were removed from trees and measured with a portable spectroradiometer (LI-1800; LI-COR, Lincoln, NE) attached by a 1800-10 quartz fiberoptic probe to an integrating sphere (model LI1800-12S; LI-COR) like the method described by Mesarch et al. (1999). A leaf disc was excised from each leaf with a cork borer (285 mm^2) and clamped into the sample port with the upper side facing the sphere interior on the sphere wall where a 165-mm^2 circular area was irradiated by the beam from a tungsten halogen lamp. Light reflected from the leaf was transmitted from the sphere interior through the fiberoptic to the spectroradiometer for measurement of reflected spectral radiance from 300 to 1100 nm at 1-nm intervals. Similar measurements were made for stray light caused by imperfect collimation of the lap beam and light reflected from a white reference material. Spectral reflectance was computed by subtracting stray spectral radiance from the spectral radiances reflected by the leaf and reference and then dividing leaf-reflected radiance by reference-reflected radiance. The result was multiplied by 100 to yield units of percent. The reflectance final result was averaged across the two separate scans made on each leaf disc. 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 an ultraviolet (UV)-visible spectrophotometer (UV-1601; Shimadzu Scientific Instruments, Columbia, MD) and total Chl concentration was calculated according to Lichtenthaler and Wellburn (1983).

Regression analyses of spectral reflectance and chlorophyll data. Using Visual Basic (version 6.0; Microsoft, Redmond, WA), a customized software application was developed to directly perform simple linear regression analysis and calculate root mean square error (RMSE) and r^2 between the spectral reflectance reading from 300 to 1100 nm at 1-nm intervals and Chl concentrations (Chl a, Chl b, or Chl a + b) in leaf discs. The r^2 of the reflectance versus Chl relationship for Chl a, Chl b, and Chl a + b at each wavelength was used to generate r^2 curves for predicting the actual OW for estimating Chl concentrations using reflectance. The RMSE of the reflectance versus Chl relationship for Chl a, Chl b, and Chl a + b at each wavelength was used to generate RSME curves to validate the strength of using r^2 curves for predicting the actual OW for estimating Chl concentrations.

Reflectance sensitivity analysis. Reflectance sensitivity measures changes of leaf spectral reflectance based on unit change in referential reflectance or leaf Chl concentration. The

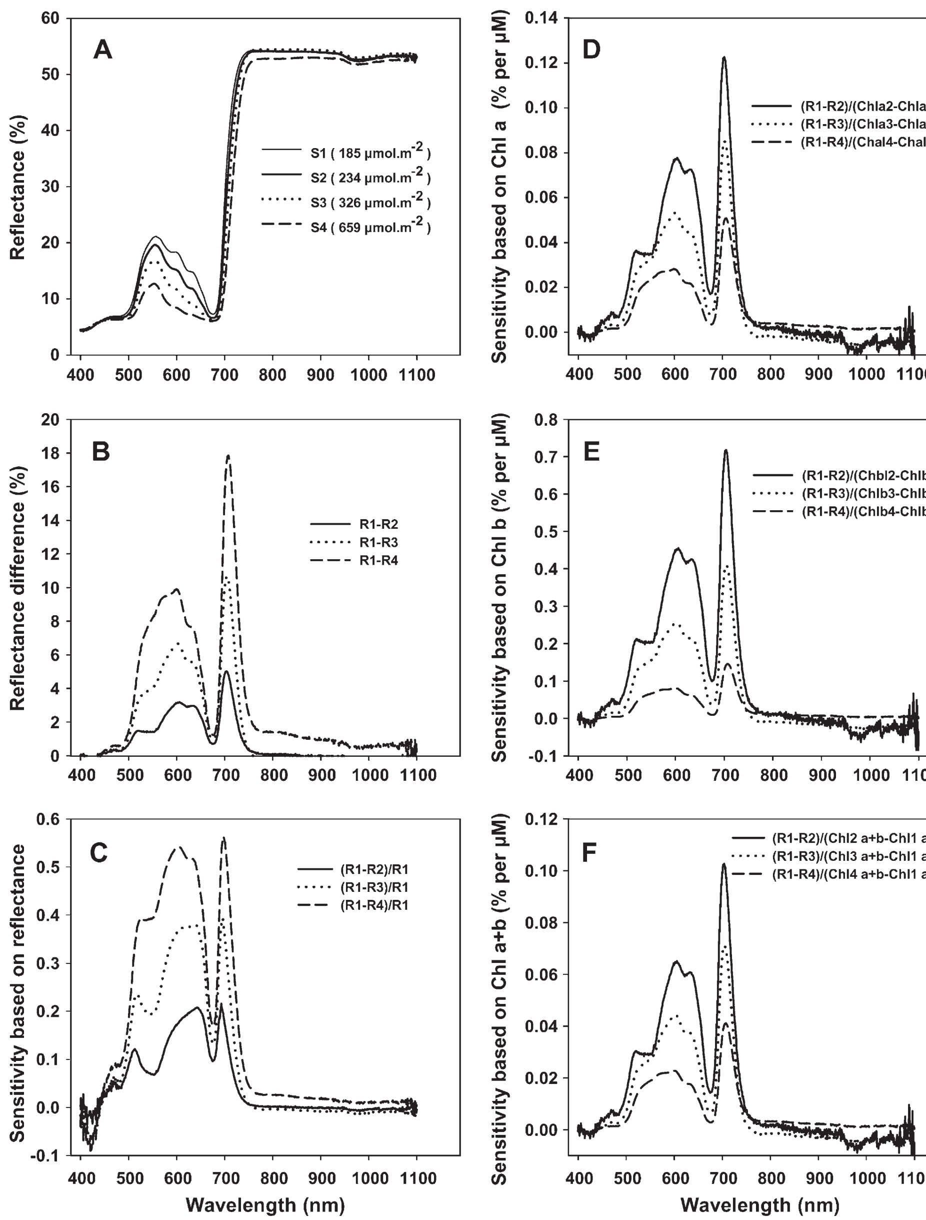

Fig. 3. Spectra of reflectance (**A**), reflectance difference (**B**), and reflectance sensitivity (**C–F**) of four UCC-1 poplar leaves (S1–S4) with different chlorophyll concentrations (Chl1–Chl4). The reflectance difference (**B**) was calculated by subtracting the reflectance of S2, S3, and S4 (R2, R3, and R4) from the reflectance of S1 (R1). The sensitivity curves based on reflectance (**C**) was calculated by dividing the reflectance difference by R1 (no unit). The sensitivity based on Chl a, Chl b, and Chl a + b (**D–F**) was calculated by dividing the reflectance difference by the difference in concentrations of Chl a, Chl b, and Chl a + b among leaf samples S2, S3, S4, and S1.

referential reflectance is the reflectance of the leaf with lowest Chl concentration in the experiment for each species, respectively. Reflectance sensitivity analysis was conducted using four single leaf samples per species selected from N fertigation treatment (S1, S2, S3, and S4) with different total Chl concentrations (i.e., Chl 1, Chl 2, Chl 3, and Chl 4) from low to high. Within a species, 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. Curves of reflectance difference versus wavelength were developed from 300 to 1100 nm at 1-nm intervals. Sensitivity curves based on referential reflectance were generated using the method described by Carter (1993, 1994) and Moran et al. (2000) by dividing the reflectance difference by the reflectance of the leaf sample with the lowest Chl concentration [i.e., (R1–R2)/R1, (R1–R3)/R1, and (R1–R4)/R1]. Sensitivity curves based on 1 $\mu mol \cdot m^{-2}$ differences in Chl (i.e., Chl a, Chl b, or Chl a + b) were generated by dividing the reflectance difference by the difference in Chl (Chl a, Chl b, or Chl a + b) concentration between the leaf sample with the lowest Chl concentration and the leaf samples with higher Chl concentrations [i.e., (R1–R2)/(Chl 2–Chl 1), (R1–R3)/(Chl 3–Chl 1), and (R1–R4)/(Chl 4–Chl 1)], respectively.

First derivative method. The first 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 first derivation of reflectance spectrum is calculated as $(R_n - R_{n-1})/(\lambda_n - \lambda_{n-1})$, where R_n is the reflectance value at wavelength n and λ_n is the wavelength.

Reflectance indices development and comparison. To evaluate the selected OW in Chl assessment, the indices of single OW, OW with one near infrared (NIR) wavelength from 750 to 1100 nm as the form of simple ratio (SR) and normalized difference vegetation index (NDVI) were developed and compared with the recommended indices for foliar Chl and/or greenness assessment. A SR is calculated as the ratio of two single wavelengths; which is also called vegetation index if the ratio is between NIR and red wavelengths of the spectrum (Richardson et al., 2002). A NDVI is strongly correlated with leaf Chl concentration (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. After indices developed, the best-fit regression (including simple linear, quadratic and polynomial regressions), r^2 and RMSE were calculated to evaluate the indices developed.

Results and Discussion

Effect of different nitrogen application on leaf chlorophyll concentration. Twice weekly, different

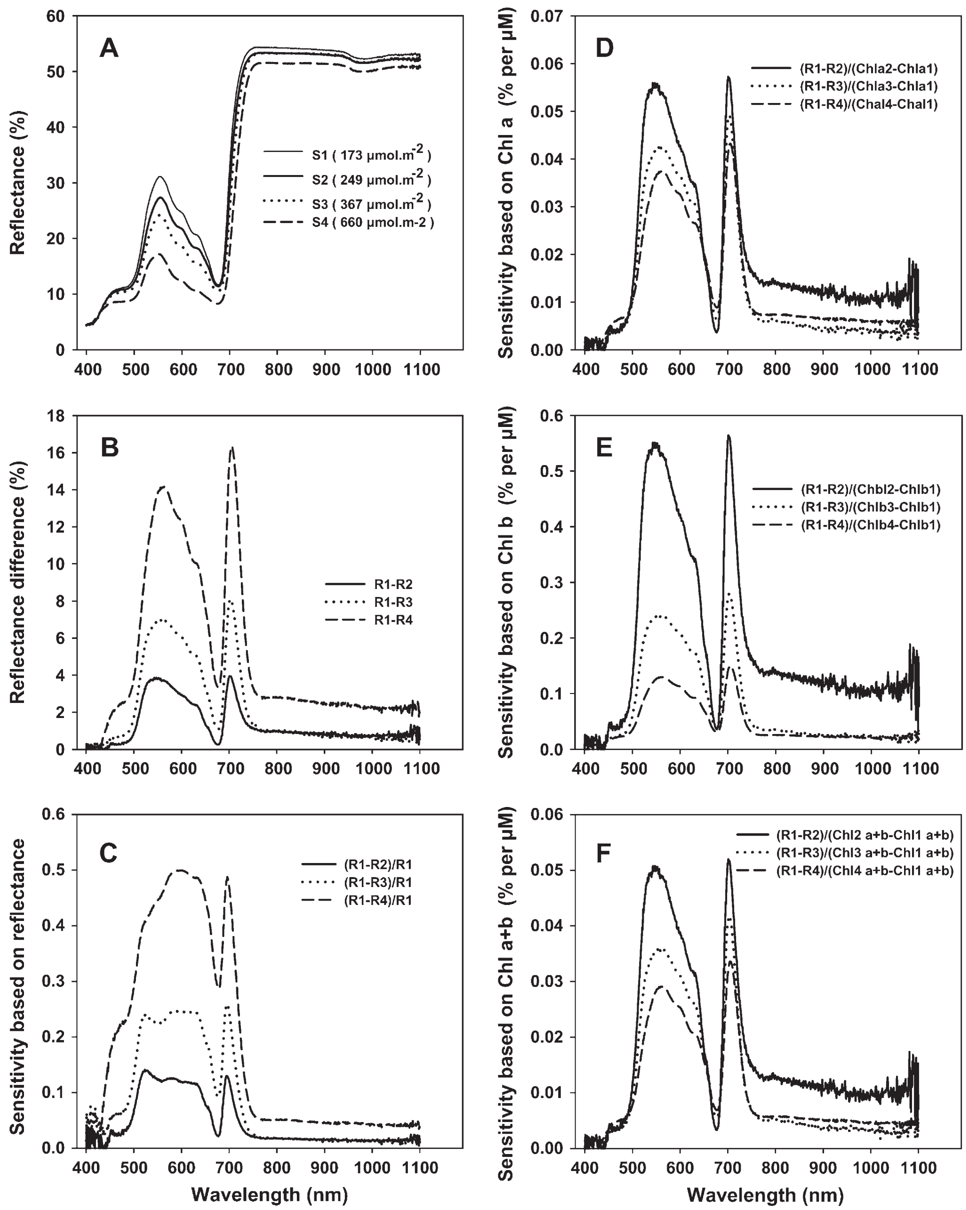

Fig. 4. Spectra of reflectance (**A**), reflectance difference (**B**), and reflectance sensitivity (**C–F**) of four 'Nonpareil' almond leaves (S1–S4) with different chlorophyll concentrations (Chl1–Chl4). The reflectance difference (**B**) was calculated by subtracting the reflectance of S2, S3, and S4 (R2, R3, and R4) from the reflectance of S1 (R1). The sensitivity curves based on reflectance (**C**) was calculated by dividing the reflectance difference by R1 (no unit). The sensitivity based on Chl a, Chl b, and Chl a + b (**D–F**) was calculated by dividing the reflectance difference by the difference in concentrations of Chl a, Chl b, and Chl a + b among leaf samples S2, S3, S4, and S1.

amounts of N application (0, 2.5, 5, 7.5, 10, or 20 mM) in fertigation solution significantly increased Chl concentration in the leave of apple, poplar, and almond from treatment to treatment within the same species, respectively (Table 1). These wide ranges of Chl concentrations in leaves of different treatments provided the ideal material used for testing the methodology of reflectance Chl nondestructive measurement.

Regression analyses of spectral reflectance and chlorophyll data. The r^2 is a measure of goodness of -fit of regression and a summary measure of regression accuracy (Chatterjee et al., 2000). Many studies used regression r^2 to evaluate the accuracy of indices for Chl assessment; only few studies have used r^2 for Chl-related waveband identification (Carter and Spiering, 2002; Gitelson et al., 2003; Read et al., 2002). The r^2 curves (Fig. 1) showed that maximum (peak) r^2 values fell in three regions: ultraviolet (380 to 440 nm), visible green (520 to 600 nm), and red edge (690 to 740 nm) (Table 2). The peak r^2 values in the red edge and green regions for Chl a, Chl b, and Chl a + b were much larger than the peak r^2 values in the ultraviolet region for all genotypes, indicating that OW selected from these two regions has a higher accuracy over the OW selected from the ultraviolet region. Peaks with larger r^2 values had the smaller RMSE, validating that simple linear regression r^2 was a reliable parameter for selecting OW for Chl assessment. By comparing the result of simple linear regression r^2 with multiple and polynomial regression r^2 in identifying OW (results not shown), we found using simple linear regression r^2 is better for OW identification, because both multiple and polynomial regressions either generated some nonmeaningful r^2 peaks or eliminated some important peaks r^2 as well as shifted the OW either to higher or lower wavelengths than actual OW. This was different from the result of Carter and Spiering (2002) in which they used both simple linear and quadratic regression r^2 to identify OW for Chl assessment and found quadratic regression r^2 is better.

The largest r^2 value from simple linear regression and the corresponding OW (for Chl a, Chl b, or Chl a + b) varied among species (Table 2). Moreover, the maximum r^2-related OW for measuring different Chl (Chl a, Chl b, and Chl a + b) within the leaves of the same species were also different. The maximum r^2 value and the corresponding wavelengths of Chl a + b were between the maximum r^2 and the corresponding wavelengths of Chl a and Chl b but tended to be closer to the maximum r^2 and the corresponding wavelength of Chl a, respectively (Table 2). These results indicate that simple linear regression can be used to identify the proper OW for assessing concentrations of specific Chl types (Chl a, Chl b, and Chl a + b) in plant species tested. The wavelengths associated with largest r^2 values for different genotypes fall in three narrow regions: ultraviolet (380 to 440 nm), green (520 to 600 nm), and red edge (690 to 740 nm) (Fig. 1A–C; Table 2). A common OW from an overlapping region from either green (550 to 580 nm) or red edge (700 to 730 nm) could be used to assess Chl across species, although it is not as accurate as using the peak OW derived for each species.

Reflectance sensitivity analysis. The original reflectance spectra for apple (Fig. 2A), poplar (Fig. 3A), and almond (Fig. 4A) leaves showed only one reflectance peak in the green region of 520 to 600 nm. Reflectance difference curves derived

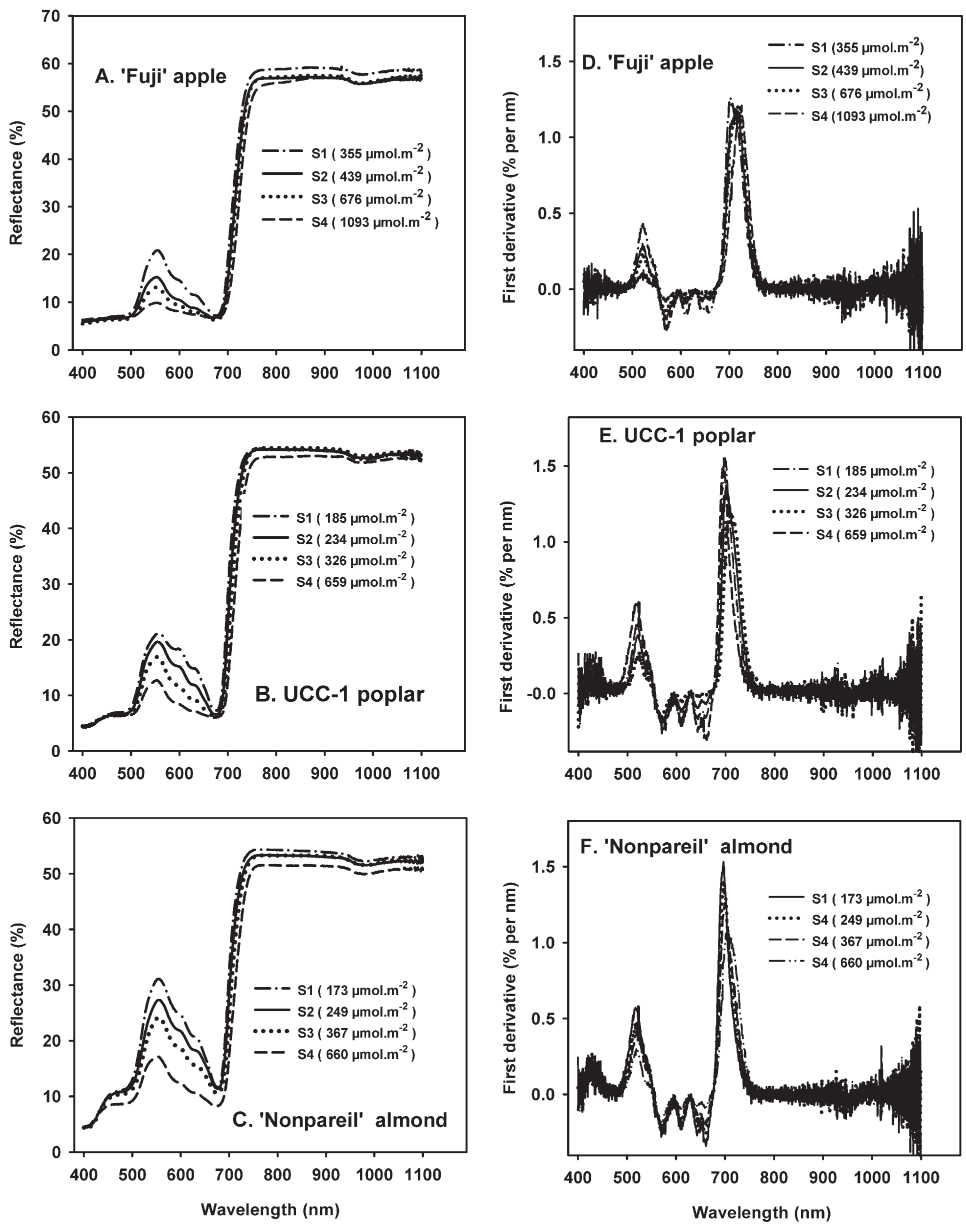

Fig. 5. The reflectance (**A–C**) and the first derivative (**D–F**) spectra of four leaves (S1–S4) with different chlorophyll concentrations for apple, poplar, and almond, respectively.

from the original reflectance spectra showed two peaks: one in the red edge region (700 to 730 nm) and the other in the green region (530 to 600 nm) (Figs. 2B, 3B, and 4B). The curve of reflectance sensitivity based on both referential reflectance (Figs. 2C, 3C, and 4C) and 1 μM·mol·m^{-2} difference in Chl a, Chl b, or Chl a + b (Figs. 2D–F, 3D–F, and 4D–F) had similar peak trends as the curve of reflectance difference, indicating that both reflectance difference and reflectance sensitivity can be used for selecting the OW for Chl assessment. Reflectance sensitivity was reported closely associated with Chl concentration and has been used to identify stress-sensitive wavelength (Carter 1993, 1994; Moran et al., 2000). When the difference in reflectance between samples was caused by the difference in Chl concentration, reflectance sensitivity based on both referential reflectance and unit difference in leaf Chl concentration had a similar result in identifying OW for Chl assessment (Figs. 2C–F, 3C–F, and 4C–F). When the difference in reflectance between samples was caused by other factors (e.g., leaf texture, and so on), reflectance sensitivity based on referential reflectance may be different from reflectance sensitivity based on differences in Chl concentration. On either condition, using reflectance sensitivity based on differences in Chl concentration is better than using reflectance sensitivity based on referential reflectance, because it can ensure the difference in spectral reflectance is caused by differences in Chl concentration.

First derivative method. The first derivative is a useful tool in characterizing or discriminating one spectral band overlapped by other bands with different bandwidths (Dixit and Ram, 1985). The first derivative of leaf reflectance spectra has been used widely and successfully to assess plant stress and to identify Chl-related wavelength in the red edge for Chl-related indices development (Curran et al., 1990; Gitelson et al., 1996a, 2003; Richardson et al., 2002), but no reports have described how peak shifts caused by first derivative transformation influence the accuracy of OW identification and Chl assessment. We found that the first derivative transformation of reflectance spectra (Fig. 5A–C) changed the original peak form by either generating some nonmeaningful peaks or eliminating some important peaks that might be Chl-related (Figs. 5D–F and 6A–C). After the first derivative transformation, the transformed reflectance spectra contained five peaks (Fig. 5D–F). Only one of these five peaks on the first 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. Within the same species, the OW selected by using the first derivatives for leaves with different Chl concentrations were slightly different. For example, the variation of OW selected for leaves with different Chl concentration in the region of red edge using the first derivative method was 23 nm (703 to 726 nm) in 'Fuji' apple leaves with Chl concentration 355 to 1093 µmol·m^{-2}, 20 nm (695 to 715 nm) in poplar leaves with Chl concentration 185 to 659 µmol·m^{-2}, and 16 nm (697 to 713 nm) in almond leaves with Chl concentration173 to 660 µmol·m^{-2} (Fig. 5D–F). This variation is big enough to impair the ability of the first derivative method to accurately identify OW.

Comparisons of r^2, reflectance sensitivity, and first derivative methods. There were two Chl-sensitive peaks (one in the visible green region and the other in the red edge region) identified in the curves of both reflectance sensitivity and r^2, but only one (the red edge region) in the first derivative curve found in all three plant species tested (Fig. 6A–C). Within the same species, the corresponding OW for the apexes of the curves obtained by different methods was also different. The OW in the red edge region identified by the r^2 method for 'Fuji' apple, poplar, and almond were 720, 720, and 710 nm, respectively; the OW identified by the method of reflectance sensitivity for the same samples were 717, 708, and 705 nm, respectively; and the OW identified by the first derivative method for the same samples were at 726, 713, and 702 nm, respectively.

OW obtained by using reflectance sensitivity or first derivative were shifted either at higher or lower wavelengths than the

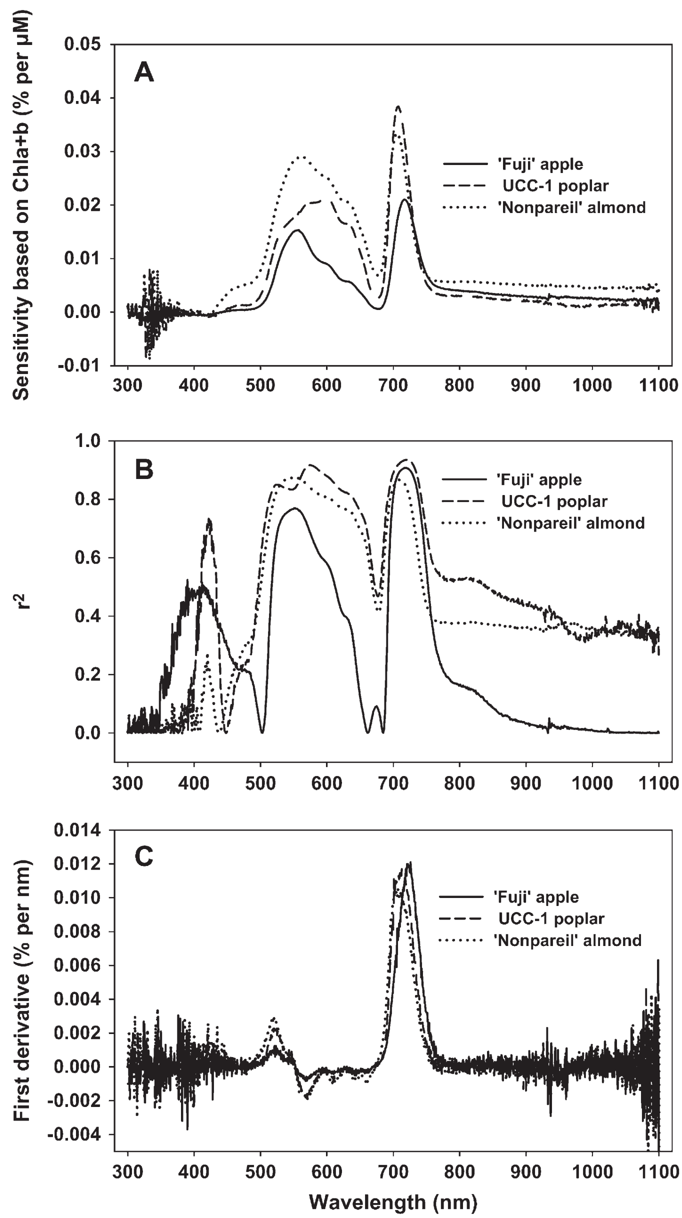

Fig. 6. Comparison of optimum wavelengths (peaks) obtained by reflectance sensitivity, r^2, and first derivative used for assessing chlorophyll (Chl) concentrations in leaves of apple, poplar, and almond. (**A**) Reflectance sensitivity curves developed by dividing reflectance difference by the difference 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**) r^2 for the relationship between total Chl concentration and reflectance values (n = 72 leaves per species).

Table 3. Best-fit coefficient of determination (r^2) and root mean square error (RMSE) of indices developed using optimum wavelengths and the indices published in accessing total chlorophyll in poplar and apple leaves.

Species	Indices	r^2	RMSE($\mu g \cdot m^{-2}$)	References
UCC-1 poplar	$R_{550-585}{}^{z}{}_{(575)}{}^{y}$	0.855 b**–0.927 c***[x]	36.30–54.77	
	$R_{700-740(720)}$	0.856 b**–0.945 c***	33.48–54.89	
	$R_{750-1100}/R_{550-585}$	0.931 c***–0.952 c***	30.16–40.56	
	$R_{750-1100}/R_{700-740}$	0.941 c**–0.961 c***	29.88–37.22	
	$(R_{750-1100}-R_{550-585})/(R_{750-1100}+R_{550-585})$	0.925 c***–0.953 c***	30.24–42.88	
	$(R_{750-1100}-R_{700-740})/(R_{750-1100}+R_{700-740})$	0.924 c***–0.959 c***	28.76–42.02	
	R_{750}/R_{700}	0.923 c***	36.88	Gitelson et al., 1996a, 1996b
	R_{750}/R_{550}	0.862 b**	54.89	Gitelson et al., 1996b
	R_{710}/R_{760}	0.940 c***	33.29	Carter, 1993, 1994
	R_{695}/R_{760}	0.832 b**	54.89	Carter, 1993, 1994
	R_{605}/R_{760}	0.868 b**	41.20	Carter, 1993, 1994
	R_{800}/R_{675}	0.609 b*	76.45	Blackburn, 1998
	R_{800}/R_{650}	0.807 b**	76.67	Blackburn,1998
	R_{709}/R_{850}	0.939 c***	34.40	Carter and Spiering, 2002
	$(R_{800}-R_{700})/(R_{800}+R_{700})$	0.904 c***	38.20	Gietelson and Merzlyak, 1994
	$(R_{800}-R_{680})/(R_{800}+R_{680})$	0.640 b*	75.10	Blackburn, 1998
	$(R_{750}-R_{675})/(R_{750}+R_{675})$	0.486 a NS	78.24	Gamon and Qiu, 1999
	$(R_{750}-R_{680})/(R_{750}+R_{680})$	0.481 a NS	78.88	Richardson et al., 2002
'Fuji' apple	$R_{540-580\ (552)}$	0.775 c**–0.799 c**	128.45–145.88	
	$R_{700-740(720)}$	0.863 c**–0.937 d***	65.80–90.32	
	$R_{750-1100}/R_{540-580}$	0.883 c**–0.948 d***	60.60–95.43	
	$R_{750-1100}/R_{700-730}$	0.913 d***–0.965 d***	57.11–72.39	
	$(R_{750-1100}-R_{540-580})/(R_{750-1100}+R_{540-580})$	0.827 c**–0.941 d***	60.60–110.91	
	$(R_{750-1100}-R_{700-730})/(R_{750-1100}+R_{700-730})$	0.912 d***–0.950 d***	59.01–72.88	
	R_{750}/R_{700}	0.861 c**	90.87	Gitelson et al., 1996a
	R_{750}/R_{550}	0.852 c**	92.10	Gitelson et al., 1996a
	R_{710}/R_{760}	0.935 d**	64.85	Carter, 1993, 1994
	R_{695}/R_{760}	0.734 b*	148.88	Carter, 1993, 1994
	R_{605}/R_{760}	0.503 a NS	195.45	Carter, 1993, 1994
	R_{800}/R_{675}	0.484 a NS	207.71	Blackburn, 1998
	R_{800}/R_{650}	0.495 a NS	200.32	Blackburn, 1998
	R_{709}/R_{850}	0.929 d***	70.61	Carter and Spiering, 2002
	$(R_{800}-R_{700})/(R_{800}+R_{700})$	0.842 c**	108.43	Gietelson and Merzlyak, 1994
	$(R_{800}-R_{680})/(R_{800}+R_{680})$	0.673 ab*	162.34	Blackburn, 1998
	$(R_{750}-R_{675})/(R_{750}+R_{675})$	0.667 ab*	168.98	Gamon and Qiu, 1999
	$(R_{750}-R_{680})/(R_{750}+R_{680})$	0.654 ab*	177.64	Richardson et al., 2002

[z]Wavelength or range of wavelengths used in the indices.

[y]Single optimum wavelength in green or red edge.

[x]NS, *, **, ***Nonsignificant or significant at $P < 0.05$, 0.01, or 0.001, respectively; r^2 followed by the same letter within a column of the same species are not significantly different ($P < 0.05$, Fisher's z test, n = 72).

actual OW obtained using the r^2 method. For example, the OW identified for poplar by regression r^2 and the first derivative and reflectance sensitivity are 720, 708, and 713 nm, respectively. There was no consistent trend that could be used for predicting which method would obtain higher or lower values. The high or low OW specified by reflectance sensitivity or first derivative curves could reduce the accuracy in determining the OW for Chl assessment. However, based on differences in Chl concentration, the OW selected by using reflectance sensitivity can ensure reflectance differences were caused by differences in Chl concentration, which was more accurate and meaningful than selected by using the first derivative. Furthermore, we found that the OW determined by reflectance sensitivity does not vary within the same plant genotype, whereas the OW selected using the first derivative varied both within and between genotypes (Figs. 2D–F, 3D–F, 4D–F, and 5D–F).

Indices comparison. Published indices have been developed based on one or two Chl-sensitive wavelengths and one Chl-insensitive wavelength (750 to 1100 nm) to increase indices accuracy (Gitelson et al., 1996a, 1996b; Richardson et al., 2002). Our result showed that even just single wavelength indices if developed with OW from red edge (700 to 730 nm) or green (550 to 580 nm) have larger r^2 and smaller RMSE than those published indices developed without OW (Table 3). The accuracy (r^2 and RMSE) can be further improved if the indices are developed by using one OW and one Chl-insensitive NIR wavelength (750 to 1100 nm) in the form of R_{NIR}/R_{OW} or $(R_{NIR} - R_{OW})/(R_{NIR} + R_{OW})$ (Table 3). In earlier published investigations, reflectance wavelengths from 670 to 680 nm were used for Chl assessment (Merzlyak et al., 2003). Although the indices developed with wavelengths 670 to 680 nm showed good sensitivity and linearity at low Chl concentrations, they became rapidly saturated and less accurate with an increase in Chl concentration over 100 to 150 $\mu g \cdot m^{-2}$ (Buschmann and Nagel, 1993; Gitelson and Merzlyak, 1994; Gitelson et al., 2003). Our results showed that indices developed using OW in either the green (540 to 580 nm) or red edge (700 to 730 nm) region had higher reflectance sensitivity and can be used across a wider range of Chl concentrations (160 to 1188 $\mu mol \cdot m^{-2}$) than those indices developed using wavelength from 670 to 680 nm (Figs. 2, 3, and 4). Indices developed with the OW from the red edge region (700 to 730 nm) are more robust to a wide range of carotenoid and anthocyanin and can be used to assess Chl in both anthocyanin-containing and anthocyanin-free species, whereas indices developed with the OW in the green region (550 to 580 nm) are influenced by the existence of anthocyanins and are better used for anthocyanin-free species (Gitelson et al., 2001; Merzlyak et al., 2003).

Conclusions

Simple linear regression r^2 combined with reflectance sensitivity and/or first derivative analysis was proven to be a reliable method for identifying OW for Chl measurement. Based on this method, two optimum wavebands that had larger r^2, smaller RMSE, and higher reflectance sensitivity were found in red edge (700 to 730 nm) and green (550 to 580 nm) regions, which can be used as common OW for Chl reflectance assessment in poplar, apple, and almond leaves tested. Single wavelength indices if developed with OW from either red edge or green were even more accurate than those more wavelength indices that developed without using OW. The accuracy of indices can be further improved if developed by using one OW and one Chl-insensitive wavelength from 750 to 1100 nm in the form of R_{NIR}/R_{OW} or $(R_{NIR} - R_{OW})/(R_{NIR} + R_{OW})$.

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