Fluorosensing of RuBisCO and Chlorophyll Contents in Common Buckwheat Leaf

Mikio SEKINUMA and Naoto INOUE
Faculty of Agriculture, Shinshu University,
Minamiminowa 8304, Nagano 399–4598, Japan

Summary

Analysis of the crop nutritional condition plays an important role in the development and application of low-input, sustainable, and environmentally friendly agricultural methods and to save limited resources. Therefore, it is necessary to have a simple and accurate method for conducting this analysis to aid decision-making. In this study, we estimated the ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) and chlorophyll contents of the leaves of the common buckwheat (*Fagopyrum esculentum* M.) by statistical analysis using the normalized differential spectral index (NDSI) and other pretreated spectral data to evaluate potential photosynthetic activity in crop breeding programs to achieve high yield. The highest determination coefficient (R^2) was recorded for chlorophyll a using the partial least-squares regression (PLS regression) method. The R^2 value related to RuBisCO contents was relatively lower and the accuracy of estimation for RuBisCO was midway between that for chlorophyll a and chlorophyll b. The R^2 value of RuBisCO/soluble protein was higher than that of RuBisCO in fresh leaves. The system of ultraviolet-induced fluorescence provides a new method for fluorosensing to evaluate the nutritional status of plants using the key biochemical that is related to the dark reaction, as well as PAM, which is related to the light reaction in photosynthesis.

Key words : *Fagopyrum esculentum*, Fluorescence, Partial least-squares regression, Photosynthesis, Plant nutrition, Ultraviolet

Introduction

Sensing of plant nutritional status plays an important role in the development and application of low-input, sustainable, and environmentally friendly agricultural techniques and to save limited resources around mountainous areas around the world. Currently, most Japanese farmers who practice precision farming perform a visual inspection of leaf color and use a color chart to estimate the nitrogen content of rice and to decide on the timing of top dressing application. Some farmers also use a SPAD meter to measure the chlorophyll content of leaves (Kitagawa *et al*., 1987). In an application of remote sensing, reflectance spectroscopic analysis has been used to estimate the chlorophyll content in leaves (Sims and Gamon, 2002) and also to measure the protein content, chlorophyll content, and biomass in rice using the hyper spectral data (Inoue *et al*., 2002). Furthermore, photo inhibition and photon quantum yield can be estimated by analyzing fluorescence-induced pulse light irradiation under daylight conditions, for which application of pulse amplitude modulated fluorometry (PAM) has been extended to the fields of plant ecophysiology and agricultural science (Schreiber *et al*., 1986). In addition to measuring the light reaction of photosynthesis, this study focused on the dark reaction and on nondestructive measurements.

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The ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) content limits the photosynthetic rate of C_3 plants. RuBisCO is the key enzyme that synthesizes CO_2 or O_2 in the initial reaction of the Calvin-Benson cycle (Farquhar *et al*., 1980). C_3 plants commonly possess large amounts of RuBisCO to effectively synthesize CO_2 because the carboxylase activity compensates for the oxygenase activity, and RuBisCO also catalyzes photorespiration and decreases the photosynthetic rate (Buchanan *et al*., 2000). Therefore, the convenient and quick estimation of RuBisCO content as the key material of the dark reaction can allow us to evaluate the potential activity of crop photosynthesis. We can use this information to increase the crop photosynthetic rate and to decrease the management cost and labor by monitoring the related environmental and physiological factors.

With respect to buckwheat (*Fagopyrum esculentum* M.), only a few research reports regarding the estimation of photosynthetic rates in the plant ecophysiological field are available in the literature. Sugimoto *et al*. (1999) reported that the maximum rate of net photosynthesis in the common buckwheat leaf occurs at approximately 20-30°C, and Gaberscik *et al*. (2002) reported that the potential and actual photochemical efficiencies of PSII were not affected by enhanced UV-B radiation. In the breeding system of common buckwheat, sink size was examined an effort to improve yield by increasing its flower fertilization rate (Inoue *et al*., 2002). The next challenge will be to increase the source size, as well as the sink size.

Our previous work (Sekinuma and Inoue, 2013) described our system of measurement of UV-induced fluorescence (SMUF) and described the use of hotspots of fluorescence for estimating RuBisCO and chlorophyll content. These hotspots can be induced by UV light (Cerovic *et al*., 1999). In this study, we estimated the RuBisCO and chlorophyll content of the common buckwheat leaves by performing a statistical analysis of the normalized differential spectral index (NDSI) and by using other pretreated spectral data to evaluate potential photosynthetic activity in crop breeding programs to achieve high yield.

Materials and Methods

Plant materials

Common buckwheat (variety Shinano No. 1) was grown in the experimental field at the Education and Research Center, Alpine Field, Shinshu University, in Nagano Prefecture, Japan (35°51′N, 137°56′E, 740 m above the sea level) from August to October 2009. The soil of the experimental site was classified as an Andosol using the FAO/UNESCO system, and the soil texture was a fine sandy loam according to the international system. Sampling times were set from 10 : 00 to 15 : 00 h during the flowering period. Four expanded leaves on each main stem were randomly selected for analysis. The fluorescence was measured at the center of each leaf after pretreatment under dark conditions for 30 min at 25°C on September 10, 2009.

Measurement of fluorescence induced by a UV lamp

We developed the SMUF for the detection of fluorescence intensity in leaves and the analysis of the spectrum (Sekinuma *et al*., 2013). In this system (Fig. 1), a fresh leaf was excited using light from a xenon lamp (LAX101 ; Asahi Spectra Co., Ltd., Japan). The light was passed through a 370 nm glass filter (band-pass filter XBPA370 ; Asahi Spectra Co., Ltd., Japan) and quartz fiber. The

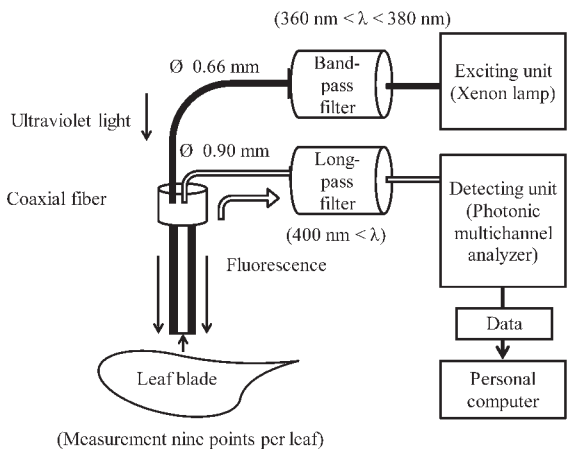

Fig. 1. UV-induced measurement system developed in the experiment.
λ : Wavelength.

emitted fluorescence, which was guided through a long-pass filter for cutting off wavelengths below 400 nm (XUL0400 ; Asahi Spectra Co., Ltd., Japan), was detected using a photonic multichannel analyzer (PMA-11 ; Hamamatsu Photonics, Japan), and wavelengths from 400 nm to 900 nm were partitioned. The distance between the edge of the fiber and the leaf sample was adjusted to approximately 4 mm. The fluorescence was detected 25 times for 0.5 second in each leaf sample (Fig. 2). For each leaf sample, nine measurements were made from different points on the leaf. The recorded data were analyzed using a personal computer to calculate the moving average, and raw fluorescence spectra were obtained.

Fig. 3 shows the UV-IF spectra of buckwheat (cv. Shinano No. 1) and rice (cv. Koshihikari) for preliminary testing. It was difficult to detect the peak of the spectrum from 400 to 550 nm that represented the protein compounds of the common buckwheat. Therefore, the statistical data treatment in the spectrum analysis was needed to estimate the RuBisCO and chlorophyll contents.

Determination of chlorophyll, RuBisCO, and soluble protein content

The SPAD data of the leaves were collected using a portable chlorophyll meter (SPAD-502 ; Konika Minoruta Co., Osaka, Japan), after which we measured the fluorescence. Next, leaf disc samples of 7 mm in diameter were removed and frozen in liquid N_2. To measure the chlorophyll content, three of the discs were soaked in 96% ethyl alcohol in the dark for 2 days for extraction of leaf color. After the pigments had been extracted, the contents of chlorophyll a and b in the alcohol solution were determined by absorbance at wavelengths of 649 and 665 nm, respectively, using a spectrophotometer (AE-350, Erma. Inc., Japan), according to the method described by Wintermans and de Mots (1965).

For analysis of the soluble protein content, six leaf discs were crushed in a mortar with liquid N_2, added to a chilled extraction buffer [100 mM NaH_2PO_4/Na_2HPO_4 buffer (pH 7.0), 1 mM phenyl methanesulfonyl fluoride, 1% (c/v) 2-mercaptoethanol, and 1% (m/v) insoluble polyvinyl polypyrrolidone], and ground. The solutions were placed in Eppendorf tubes and centrifuged at 12,000×g, 4°C, for 5 min. The supernatant liquid was divided and the Bradford reagent (Bio-Rad, USA) was added (Bradford, 1976). The amount of soluble protein in the sample was determined on the basis of absorbance at 595 nm by using a spectrophotometer. The concentrations (w/w) of RuBisCO in the soluble protein were quantified by SDS-polyacrylamide gel electrophoresis, according to the method described by Makino *et al*. (1985).

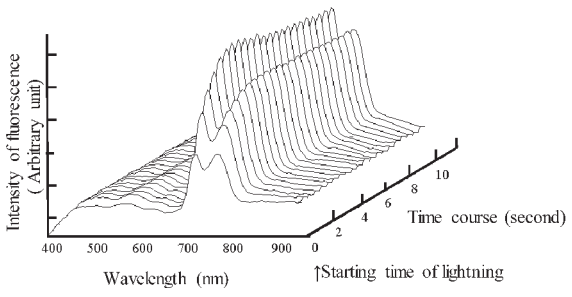

Fig. 2. UV-induced fluorescence spectra from a common buckwheat leaf.
Excitation : 370 nm, detection : 400-900 nm, exposure time : 0.5 second, repeat : 25, moving average : 5 points for 25 nm, total measurement time : 12.5 second.

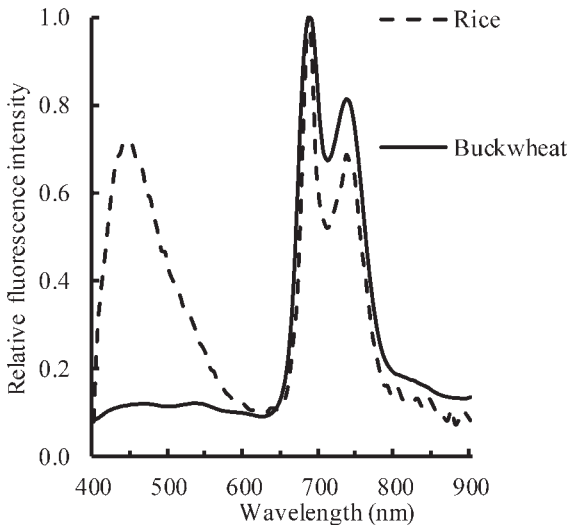

Fig. 3. UV-IF spectra of buckwheat and rice.
The relative intensity means the value of fluorescecnce to that of 685 nm.

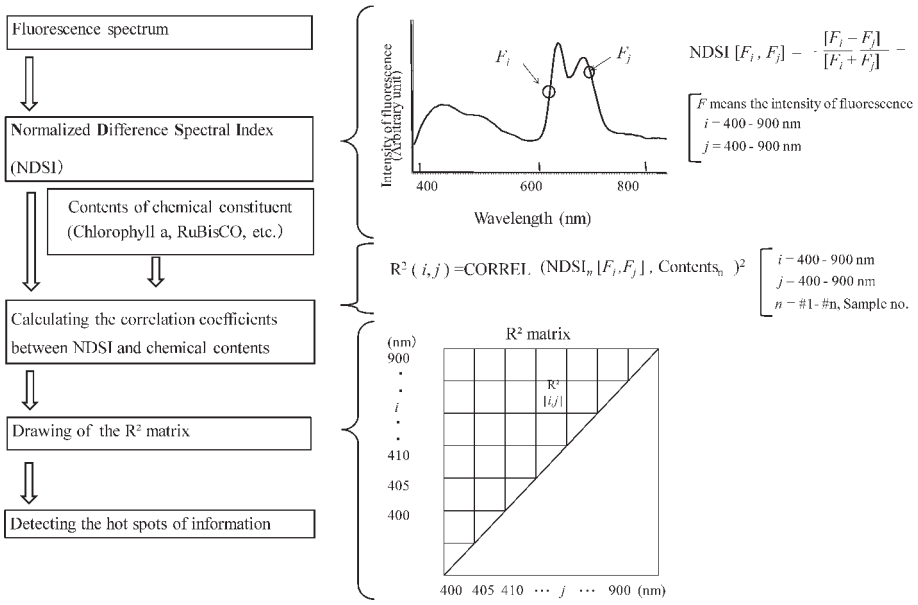

Fig. 4. Procedure for calculating the matrix of the determination coefficient (R^2) between NDSI and the chemical contents.

Analysis of the relationship between fluorescence and the chemical constituents

The 1st derivative spectra were obtained from the raw fluorescence spectra. The NDSI was calculated to achieve an accurate spectral analysis (Fig. 4). The normalized difference vegetation index combines two spectral bands (Qi *et al*., 1994), whereas the NDSI uses all combinations of the information from two separate wavelengths (Inoue *et al*., 2008). The NDSI was defined to detect hotspots of information in the fluorescence data, which is related to the chemical contents as follows:

$$\mathrm{NDSI}\ [F_i,\ F_j]=\frac{F_i-F_j}{F_i+F_j};$$

in which *Fi* and *Fj* are the fluorescence intensities of the *i* and *j* wavelength bands, respectively.

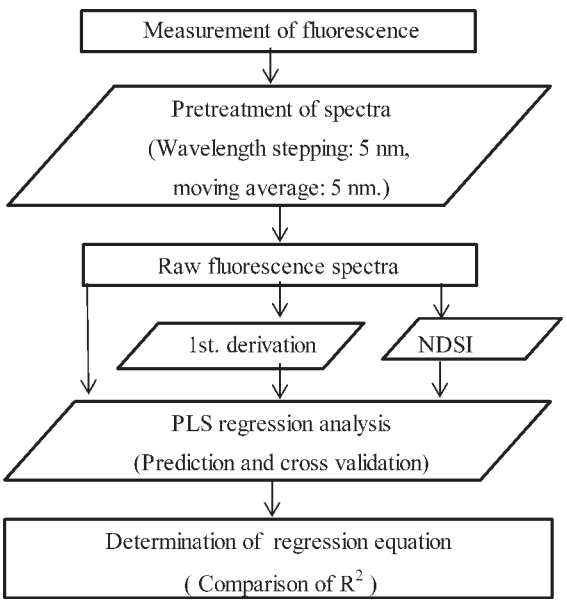

Fig. 5. Algorithm for data processing.

In the present study, all possible two-band combinations of spectral indices were obtained, ranging from 400 nm to 900 nm. The contributions were constructed in the form of a matrix linkage according to Yao *et al*. (2010). Table 1 shows the hotspots in the matrix of the coefficient of determination (R^2 matrix) for the linear relationship between the NDSI and chemical contents.

Partial least squares (PLS) regression analysis

The PLS regression analysis was adopted to estimate the chemical constituent related to the photosynthetic activity as determined by our fluorescence data (Fig. 5). The restricted NDSI data that contained the information on these hotspots were used to compress the huge quantity of NDSI data on the independent variables (Table 1). The PLS analysis was calculated using a spreadsheet program (Microsoft Excel 2010, Co., USA) and a multivariate analysis program (Excel Multivariate Analysis ver. 6.0, Esumi

Table 1. Information hotspots of chemical constituents.

Chemical constituents	Distribution of information hot spots in NDSI matrix	
	X axis (nm)	Y axis (nm)
TC	705,725	725,750
SP	600-635, 670-690, 715-720	600-650, 695-705, 720-740
SP and RuBisCO	600-760	610-750
RuBisCO	660-720	670-710

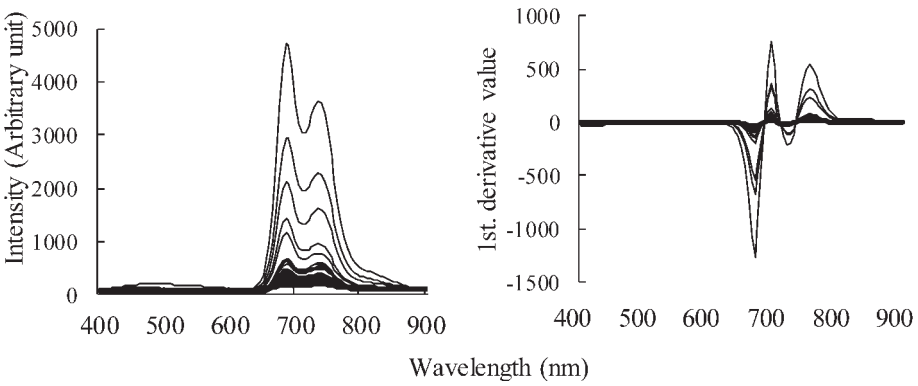

Fig. 6. UV-induced fluorescence spectra of the buckwheat leaf.
n=30.

Co., Tokyo). R^2 and Akaike's Information Criterion (AIC) were used to evaluate the PLS equation. AIC is one of criteria to judge the performance, the goodness of fitting, and the simpleness of the model. The simpleeness of model can be measured by a number of parameters and the wrong model has commonly several parameters in the equation. Therefore, we evaluated R^2 and AIC, and the lower value of the AIC meant that it is a relatively better model for unknown samples, as well as for these samples in constructing a PLS regression.

Results

There was a significant difference in band fluorescence intensity among samples using wavelengths within the range of 650 to 800 nm. Maximum fluorescence intensity was 30-fold higher than the minimum (Fig. 6). In the 1st derivative data, there was a 100-fold difference in fluorescence intensity at approximately 670 nm in the spectrum.

The chemical analysis values that were used for statistical analysis are shown in Table 2. The average level of RuBisCO was similar to that of chlorophyll a. The coefficient of variance values of the constituents related to RuBisCO and total chlorophyll/soluble protein (TC/SP) were higher than the values related to the chlorophyll a and b contents. The higher R^2 value of PLS regression analysis was observed in the NDSI data sets compared to the data set of raw spectra or the 1st deviated spectra. The lower R^2 value was observed under SP, and the accuracy of estimation was lower. However, the R^2 value of the TC/SP was higher than that of SP. The highest R^2 value was recorded in chlorophyll a [latent variable=6 ; R^2=0.876 ; AIC=-192.8, Fig. 7 (A)]. On the other hand, the values for chlorophyll b and the ratio of chlorophyll a and chlorophyll b could not be estimated using the PLS regression method. The R^2 value related to RuBisCO contents was 0.494, and the accuracy of estimation was obtained midway between chlorophyll a and chlorophyll b [latent variable=4 ; R^2=0.494 ; AIC=-60.0, Fig. 7 (B)]. The R^2 value for RuBisCO/SP and SP/RuBisCO were higher than that of RuBisCO in the fresh leaf [latent variable=8 ; R^2=0.739 ; AIC=-101.4, Fig. 7 (C)], whereas the RuBisCO/TC values were lower than the R^2 of RuBisCO/SP [latent variable=4 ; R^2=0.460 ; AIC=4.3, Fig. 7 (D)].

Table 2. Chemical constituent of the sample in common buckwheat leaf.

Chemical constituents	Dimension	Average	S.D.*	Range		C.V.**
				Min.	Max.	
Soluble protein (SP)	g m^{-2}	1.62	0.59	0.50	2.84	36.5
Total chlorophyll (TC)	g m^{-2}	0.33	0.04	0.25	0.45	13.3
TC/SP	-	0.24	0.12	0.16	0.50	48.8
Chlorophyll a	g m^{-2}	0.23	0.03	0.19	0.28	11.7
Chlorophyll b	g m^{-2}	0.10	0.03	0.06	0.24	30.6
Chlorophyll a/Chlorophyll b	-	2.40	0.41	1.16	2.89	16.9
RuBisCO	g m^{-2}	0.22	0.11	0.08	0.47	48.3
RuBisCO/SP	-	0.14	0.07	0.10	0.18	45.2
RuBisCO/TC	-	0.67	0.30	0.30	1.06	45.2
RuBisCO/Chlorophyll a	-	0.94	0.41	0.41	1.67	43.5

n=30

*Standard deviation

**Coefficient of variance (%)

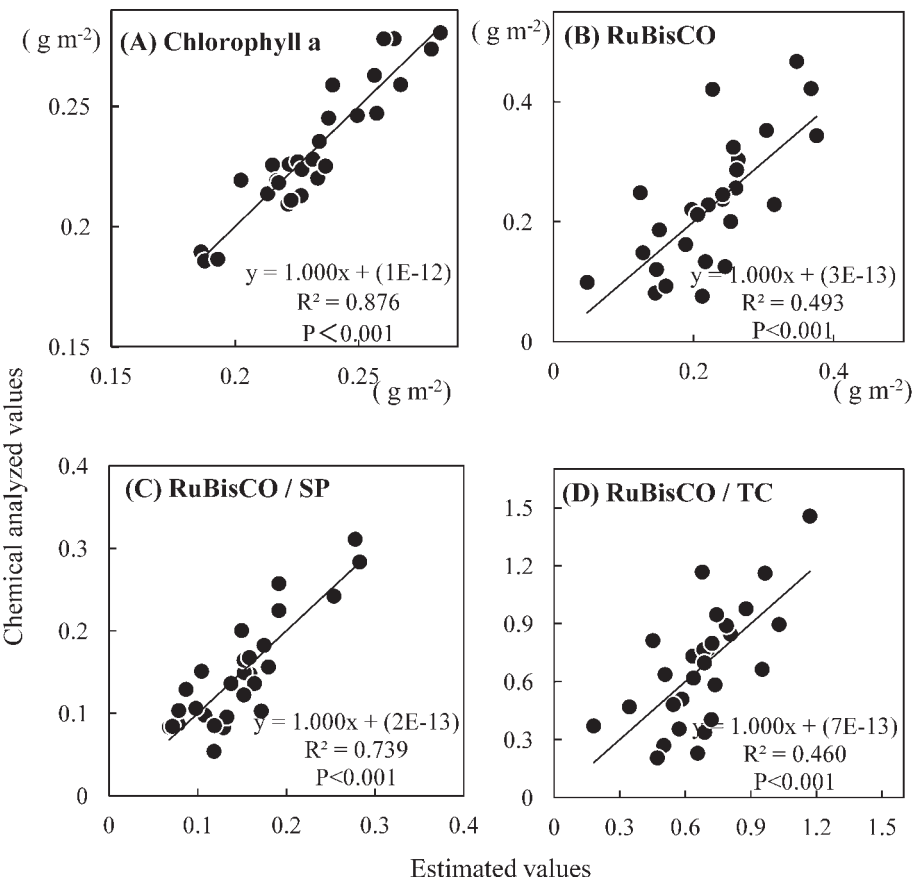

Fig. 7. Correlation between measured and calculated values by PLS.

Discussion

Evaluation of the SMUF for estimating RuBisCO and chlorophyll content

The science of evaluating plant nutrient levels in crop leaves was developed from an evaluation of leaf color using a visual color board to multispectral imaging using reflectance spectroscopy, and to fluorescence analysis, as previously mentioned. From the results of PLS regression analysis using NDSI data from the hotspot region related to chlorophyll a or RuBisCO levels, we found that the SMUF was useful in

estimating RuBisCO and chlorophyll a content. Our technique of nondestructive and simultaneous parallel analysis of the RuBisCO and chlorophyll content is the first to be reported in the field.

Saito *et al*. (2005) found that fluorescence analysis of the amount of chlorophyll a was a useful method in monitoring plant activities. They also reported that the intensity ratio of UV laser-induced fluorescence of 740 nm to 685 nm was linearly correlated to the chlorophyll a content of the leaves. In our study, the UV light-induced fluorescence and the NDSI calculation were also valuable in precisely estimating the concentration of chlorophyll a per unit leaf area in the near sensing. On the other hand, estimating chlorophyll b content by PLS regression was difficult because the R^2 values were lower, and the AIC was higher. Therefore, we hereby report that it is difficult to detect fluorescence because the absorption of UV-A light by chlorophyll b is lower than that by chlorophyll a (Mimuro *et al*., 2011). The accuracy of the estimation for the RuBisCO content significantly improved with the use of the NDSI (660-720 and 670-710 nm), which included the valuable fluorescence measurements typically used in estimating the quantum yield of photosystem II (PSII).

Chlorophyll content and RuBisCO carboxylase activity were approximately proportional to the leaf nitrogen content of wheat (Evans, 1983). In our study, a higher accuracy of PLS regression was obtained by using the NDSI data related to the fluorescence emitted from PSII. We suggest that the close relationship between RuBisCO carboxylase activity and the contents of chlorophyll involved in PSII is related to the amount of nitrogen supplied to the leaves, and that the fluorescence band emitted from PSII provides useful indirect information in this regard.

Compared to the other data processing techniques, NDSI processing provides a higher R^2 value for the characteristics related to photosynthesis. These results suggest that the NDSI processing selection of band and the PLS analysis are useful for the development of measurement equipment that is simple to operate and cost-effective.

Evaluation of dark and light reactions

Determining the ratio of RuBisCO content to chlorophyll a content is a valuable method for evaluating the photosynthetic rate of leaves (Kumagai *et al*., 2009). If the RuBisCO content of a leaf could be measured by nondestructive methods, we can then obtain physiological information on the dark reaction for fixing CO_2 and on photorespiration. In particular, indicators such as RuBisCO/TC are important in evaluating the photosynthetic rate and understanding the dynamics of the eco-physiological system. The RuBisCO content per unit leaf area will also contribute to a model for predicting the rates of photorespiration and CO_2 fixation.

The photosynthetic rate is always influenced by leaf temperature, leaf conductance, photosynthetically active radiation intensity, and CO_2 concentration. Therefore, the net photosynthesis rate will be reduced when a leaf has a lower water potential (Ortiz-Lopez *et al*., 1991). In the case of C_3 crops, leaf conductance may be estimated by measuring the leaf and air temperatures, which relate to stomatal condition (Kuraishi *et al*., 1981). The chlorophyll and RuBisCO contents accessible through nondestructive sensing indicate potential photosynthetic activity. Remote sensing can form the basis of a new dynamic model for growing crops, using data for light intensity, as well as leaf and air temperature.

Breeding objectives from morphological to physiological characteristics

We consider the SMUF as an effective approach in a conventional breeding system because it identifies physiological characteristics that influence the increase in photosynthetic rates, thus enabling the farmer to implement labor-effective procedures. Conventional and modern breeding, as represented by the "Green Revolution", have focused on the plant type, as well as high nitrogen input conditions in the field (Peng and Kush, 2003). A recent breeding objective is the improvement of the photosynthetic rate by

genetic modification to introduce the C_4 cycle to a C_3 plant, as in the case of rice. A nondestructive method of measuring key materials in the light and dark reactions of photosynthesis, as presented in this paper, will allow the development of new breeding objectives that are related to the photosynthetic metabolism in common buckwheat.

The SMUF is a new method of sensing the nutritional status of plants and diagnosing conditions for crop cultivation, which uses key materials that are involved in the dark reaction of photosynthesis, as well as PAM, which is related to the light reaction of photosynthesis. Future research studies on improving the accuracy of the measurement system by using laser excitation to effectively detect the fluorescence from protein compounds in buckwheat is thus warranted. 2-D fluorescence imaging may also be used to improve photosynthetic rates by analyzing the distribution of the pigments and enzymes that regulate these processes.

References

Bradford, M. M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem*. 72 : 248-54.

Buchanan, B. B., W. Gruissem and R. L. Jones, 2000. Biochemistry and molecular biology of plants. *The American Society of Plant Physiology*. pp. 716-728. Rockville, Maryland. USA.

Cerovic, G. Z., G. Samson, F. Morales, N. Tremblay and I. Moya, 1999. Ultraviolet-induced fluorescence for plant monitoring : present state and prospects. *Agronomie* 19 : 543-578.

Evans, J. R., 1983. Nitrogen and photosynthesis in the flag leaf of wheat (*Triticum aestivum* L.). *Plant Physiol*. 72 : 297-302.

Farquhar, G. D., S. V. Caemmerer and J. A. Berry, 1980. A biochemical model of photosynthetic CO_2 assimilation in leaves of C_3 species. *Planta* 49 : 78-90.

Gaberščik, A., M. T. Vončina, M. T. Trošt, M. Germa and L.O. Björn 2002. Growth and production of buckwheat (*Fagopyrum esculentum*) treated with reduced, ambient, and enhanced UV-B radiation. Journal of Photochemistry and Photobiology B : Biology 66 : 30-36.

Inoue, N., H. Kumagai and M. Hagiwara, 2002. Improvement of fertilization rate by mass selection in common buckwheat. *Fagopyrum* 19 : 49-53.

Inoue, Y., M. Giashudin, E. Sakaiya, K. Nakano and K. Kawamura, 2008. NDSI map and IPLS using hyperspectral data for assessment of plant and ecosystem variables : With a case study on remote sensing of grain protein content, chlorophyll content and biomass in rice. *Journal of the Remote Sensing Society of Japan* 28 : 317-330.

Inoue, Y., J. Penuelas, A. Miyata and M. Mano, 2008. Normalized difference spectral indices for estimating photosynthetic efficiency and capacity at a canopy scale derived from hyperspectral and CO_2 flux measurements in rice. *Remote Sensing of Environment* 112 : 156-172.

Kitagawa, Y., K. Okayama and T. Hirokawa, 1987. Determination of leaf color in rice plant, "Koshihikari" with a chlorophyll meter. *Bulletin of the Toyama Agricultural Research Center* 1 : 1-7.*

Kumagai, E., T. Araki and F. Kubota, 2009. Characteristics of gas exchange and chlorophyll fluorescence during senescence of flag leaf in different rice (*Oryza sativa* L.) cultivars grown under nitrogen-deficient condition. *Plant Prod. Sci*. 12 : 285-292.

Kuraishi, S., Y. Hashimoto and M. Shiraishi, 1981. Latent periods of cytokinin-induced stomatal opening in the sunflower leaf. *Plant Cell Physiology* 22 : 911-916.

Makino, A., T. Mae and K. Ohira, 1985. Photosynthesis and ribulose-1,5-bisphosphate carboxylase/oxygenase in rice leaves from emergence through senescence. Quantitative analysis by carboxylation/oxygenation and regeneration of ribulose 1,5-bisphosphate. *Planta* 166 : 414-420.

Matsushima, S., A. Matsuzaki and T. Tomita, 1970. Analysis of yield-determining process and its application to yield-prediction and culture improvement of lowland rice : CI. On a method for expressing the leaf-colour of rice plants under field conditions (1). *Jpn. J. Crop Sci.* 39 : 231-236.

Mimuro, M., T. Kakitani and H. Tamiaki, 2011. Chlorophylls : Structure, Reaction and Function, pp. 282-283. Shokabo, Tokyo, Japan.

Ortiz-Lopez, A., D. R. Ort and J. S. Boyer, 1991. Photophosphorylation in attached leaves of Helianthus annuus at low water potentials. *Plant Physiol.* 96 : 1018-1025.

Peng, S. and G. S. Khush, 2003. Four decades of breeding for varietal improvement of irrigated lowland rice in the International Rice Research Institute. *Plant Prod. Sci.* 6 : 157-164.

Qi, J., A. Chehbouni, A. R. Huete, Y. H. Kerr and S. Soroshian, 1994. A modified soil adjusted vegetation index. *Remote Sens. Environ.* 48 : 119-126.

Saito, Y., T. Matsubara, F. Kobayashi, T. Kawahara and A. Nomura, 2005. Laser-induced fluorescence spectroscopy for *in-vivo* monitoring of plant activities. *The 7th Fruit, Nut and Vegetable Production Engineering Symposium* : 699-708.

Schreiber, U., U. Schliwa and W. Bilger, 1986. Continuous recording of photochemical and non-photochemical chlorophyll fluorescence quenching with a new type of modulation fluorometer. *Photosynthesis Research* 10 : 51-62.

Sekinuma, M. and N. Inoue, 2013. Non-destructive measurement of RuBisCO and chlorophyll in common buckwheat leaf by ultraviolet-induced fluorescence. *Fagopyrum* 30. In printing.

Sims, D. A. and J. A. Gamon, 2002. Relationships between leaf pigment content and spectral reflectance across a wide range of species, leaf structures and developmental stages. *Remote Sensing of Environment* 81 : 337-354.

Sugimoto, H. and T. Sato., 1999. Summer buckwheat cultivation in the warm southwestern region of Japan. *Jpn. J. Crop Sci.* 68 : 39-44.*

Wintermans, J. F. and A. D. de Mots, 1965. Spectrophotometric characteristics of chlorophyll *a* and *b* and their pheophytins in ethanol. *Biochim Biophys Acta.* 109 : 448-453.

Yao, X., Y. Zhu, Y. C. Tian, W. Feng and W. X. Cao, 2010. Exploring hyperspectral bands and estimation indices for leaf nitrogen accumulation in wheat. *Int. J. Appl. Earth Obs.*, 12 : 89-100.

* : Japanese with English summary.