Studies on a Method for Determining the Deepness of Green and Color Chlorophyll Content of Intact Crop Leaves and Its Practical Applications

1. Principle for estimating the deepness of green color and chlorophyll content of whole leaves*

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Leaf color, especially the deepness of green color, is one of the most important visible characteristics of plants, which show varietal differences and changes due to growth conditions. Moreover, the green color of leaves has an important significance from physiological point of view, as it reflects the chlorophyll pigments contained in the leaf. In relation to this, Yamada⁴⁾ studied on the leaf color of rice plants using a reflectance method and indicated that high correlation was seen between the chlorophyll content of leaves and the leaf color, expressed as the reciprocal of percentage reflectance of visible light from the leaf surface, and that the relationship was always higher in case where the chlorophyll content was expressed on leaf area basis than in case where it was expressed on fresh weight basis. Benedict and Swidler¹⁾ reported that a quantitative relationship was shown to exist between the chlorophyll content per dry weight of soybean and Valencia orange leaves and their percentage reflectance of light of wave length 625 m μ .

The present investigation was carried out in order to find out a quick and simple method for estimating the deepness of green color of crop leaves based on the chlorophyll content, and then to contribute to the characterization of specific or varietal difference and for diagnosis of the nutritional status of the plants as well.

MATERIALS and METHODS

Leaves of *Oryza sativa* L., *Hordeum vulgare* L., *Triticum aestivum* L., *Avena sativa* L., *Secale cereale* L., *Loium multiflorum* Lam., *Lolium perenne* L., *Trifolium pratense* L., *Astragalus sinicus* L., etc. were taken from the field or pots and leaves of some garden plants were sampled from near the laboratory.

Absorbance spectra of 80 percent acetone extract of rice, barley and wheat leaves were determined by a Hitachi spectro-electric photometer, Model EPU-2A.

The spectra of whole leaves were also recorded to get the fundamental data for determining the chlorophyll content of fresh leaves. The absorbance (optical density) by the whole leaves was expressed in terms of "Attenuance" as described by Shibata³⁾ The sample leaf was cut off to a suitable size, so as to be inserted into the cell attached to the spectrophotometer and to sufficiently cover the light path of the cell holder. The leaf piece (about 1 cm $\times$ 4 cm) was erected in the cell and a few drops of water was added at its bottom. And then, the attenuation spectrum was taken by the ordinal method using one layer of filter paper, Toyo-Rohi No. 6, as the control. With respect to various leaves described above, difference attenuation was determined by subtracting the attenuation at 750 m μ of light wavelength from that at 670 m μ .

Besides, a portable chlorophyllometer was produced for a trial and tested on the relation between the readings of various living leaves and the deepness of green color or the chlorophyll contents of them.

Chlorophyll content of a small leaf area was determined by the following method as described by Osborne and McCalla²⁾. The crosssectional area of light flux in the chlorophyllometer was a half square centimeter (5 mm $\times$ 10 mm). Two or four pieces having the area were cut off from the leaf and chopped after taking the reading. The fine pieces were placed into a graduated test tube contained 5 ml of 80 percent hot ethanol and kept in the water bath at about 70°C till the pieces become bleached. After cooling the tube, the volume of extract was made up to 10 ml by 80 percent ethanol. Total chlorophyll (a plus b) content was obtained by determining both optical densities at the wavelengths of 645 and 665 m μ using the spectrophotometer.

RESULTS and DISCUSSIONS

Absorbance spectrum of leaf extract

The absorbance spectra of 80 percent acetone extracts of rice, barley and wheat leaves were

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taken for reference. The results obtained are as shown in figure 1. Distinct two peak absorbances were indicated at the wave lengths of 430 $m\mu$ and 660 $m\mu$, which seems to show mainly the specific absorbance by chlorophyll a. A flat absorbance band was observed in the range of 500 to 600 $m\mu$, and no apparent absorbance in the range beyond 700 $m\mu$. These results coincide with the absorbance spectra, already described by many investigators in regard to acetone and alcohol extracts of leaves.

Attenuance spectrum of whole leaves

The results obtained on several kinds of leaf are shown in figures 2 and 3. The features of these spectra were almost the same with each other among the leaves used in the experiment. Namely, a sharp attenuation was shown at the wave length of 670 $m\mu$ and a minimum and flat one in the band beyond 750 $m\mu$, within a limit of the wave length 500 to 800 $m\mu$. This peak attenuation band must be originated from chlorophylls in the leaf, though it shifted 10 $m\mu$ toward the longer wave length than that in the acetone extracts. The flat band, which was called in terms of "base line" by Shibata³⁾, also moved by about 50 $m\mu$ to the same side.

In the next place, attenuation spectra of rice leaves showing different degree of green color were compared, and the result was given in figure 4. According to the result, a peak attenuation was shown at the band of 673 $m\mu$, and it seems to suggest that there is a certain relationship between the attenuation at about 670 $m\mu$ and the deepness of green color. On the other hand, the base line was seen in the same band as described above, and the attenuation was not much different among the leaves regardless of the marked change in the deepness of green color of them. Similar result to that was obtained in leaves of evergreen burning bush (*Euonymus japonica* Thunb.) as shown in figure 5. In this test, a variegated yellow part of the leaf was also employed, and it was confirmed that there was no peak attenuation which was to be attributed to chlorophylls and that the base line was

Fig. 1. Absorbance spectra of acetone extract of leaves.

Fig. 2. Attenuance spectra of whole leaves(1).

Fig. 3. Attenuance spectra of whole leaves(2).

Fig. 4. Comparison of the attenuation spectra in the whole rice leaves of different deepness of green color.

Fig. 5. Comparison of the attenuation spectra in the whole leaves of different deepness of green color in *Euonymus japonica* Thunb.

Fig. 6. Comparison of the attenuation spectra in leaves of widely different coloration.

already established in the band beyond 570 $m\mu$.

An additional experiment was carried out to compare the attenuation spectra of various leaves of different colors with that of normal green leaves. The results obtained are as shown in Figure 6. In the leaves showing brownish, reddish or purplish color, relatively high and sloped band was shown in the range of 500 to 600 $m\mu$, unlike green one. The peak attenuation by chlorophylls was still discriminatable near the wave length of 670 $m\mu$, and the base line band also coincided with that mentioned above, with the exception of a brown spotted rice leaf.

From these results, it is suggested that the peak attenuation of leaves at the wave length of 670 $m\mu$ is dependent on the deepness of green color and the chlorophyll content, and the base line in the band beyond 750 $m\mu$ is due to a basic attenuation of the leaf structure, which has no relation to any pigment

contained in the leaf. Consequently, a quantitative estimation of chlorophyll content of whole leaves can be made by means of a difference attenuation, which is expressed as the balance of attenuation at 670 $m\mu$ (A_{670}) minus that at a band between 750 and 800 $m\mu$ ($A_{750-800}$) in the sample, though with a certain limit for brownish, reddish and purplish leaves.

Difference attenuation of various leaves

If the difference attenuation indicates directly the chlorophyll content per unit leaf area, it should be proportional to the leaf thickness in case where the chlorophyll content per area remains the same. To confirm this, the change in the difference attenuation according to the number of leaf layers inserted was tested using nine kinds of leaves. The results obtained are as given in table 1. The relation between the difference attenuation and the number of layers of the leaf showed changes to a certain extent. A proportional relation was, however, found in the average values of the attenuances when those leaves were grouped into two groups, deep green and moderate green, as far as the values remained less than 2.0. Thus, it is suggested that the difference attenuation of leaves is essentially in conformity to Lambert-Beer's law, notwithstanding the leaves are either translucent or almost opaque.

Moreover, the difference attenuation ($A_{670} - A_{750}$) was tested on various leaves which widely varied in the deepness of green color. The result indicates that the value of difference attenuation is able to sufficiently express the deepness of green color of

Table 1. Change in the difference attenuation according to the number of layers of leaf.

Leaf group	Name of plant	Number of layers		
		1	2	3
Moderate green	<i>Oryza sativa</i>	0.91	1.38	2.12
	<i>Avena sativa</i>	0.90	1.90	2.27
	<i>Prunus donarium</i>	0.60	1.49	2.12
	<i>Acer palmatum</i>	0.61	1.40	2.07
	<i>Platanus orientalis</i>	0.88	2.17	2.54
	Average	0.78	1.67	2.22
Deep green	<i>Hordeum vulgare</i>	1.31	2.43	—
	<i>Triticum aestivum</i>	1.52	2.35	2.48
	ditto	1.34	2.22	—
	<i>Avena sativa</i>	1.41	2.51	—
	Average	1.40	2.38	—

Table 2. Difference attenuance of various leaves by spectrophotometer.

Name of plant	Age of leaf	Deepness of green color	Difference attenuance (A670—A750)
<i>Oryza sativa</i>	O	+	0.55
	M	++	0.93
	M	+++	1.30
<i>Hordeum vulgare</i>	O	++	1.20
	Y	+++	1.49
<i>Avena sativa</i>	Y	++	0.66
	O	+++	1.38
<i>Brassica napus</i>	D	—	0.10
	M	++	1.22
	O	+++	1.72
<i>Eriobotrya japonica</i>	Y	++	0.90
	O	+++	1.06
<i>Platanus orientalis</i>	Y	+	0.63
	Y	++	0.86
<i>Stauntonia hexaphylla</i>	Y	++	0.67
	O	+++	1.61
<i>Euonymus japonica</i>	Y	++	1.86
	O	+++	2.15
<i>Quercus myrsinaefolia</i>	Y	++	0.87
	O	+++	1.23
<i>Prunus donarium</i>	Y	+	0.47
	Y	++	0.68
	Y	+++	0.88

Note : Y, M, O and D indicate young, mature, old and dead leaves, respectively.

leaves as shown in Table 2.

Trial production and test of a portable chlorophyllo-meter

On the basis of these results as described above, a chlorophyllo-meter, which possesses the following conditions, was produced for a trial. (1) Light source: A 2.5 volt miniature lamp with two dry cells of 1.5 volt each. (2) Interference filters: Two filters which having each peak transmission at the wave length of 670 and 750 m μ . (3) Slit (lighting area): Half square centimeter (5 mm $\times$ 10 mm) is suitable for narrow leaves such as graminaceous plants. (4) Photocell: Cadmium sulfide cell with the highest sensitivity. (5) Meter: Micro-ammeter graduated with $-\log T$. (6) Filter adjustment circuit: Transmittant energy from both filters must be adjusted synchronizingly with the change of filter by a resistance, without changing the lighting area for the sample leaf. (7) Construction: The meter is separated into two parts which are connected with a lead. One of them is the optical system including a lamp, two filters, a sample holder and a photocell, and the other is the system including the meter and other parts which are placed in a box. The former can be held with a hand and the latter can be hanged from shoulder. The structural diagram of the meter is given in figure 7.

Fig. 7. Structural diagram of the chlorophyllo-meter.

L : Light source, F750, F670 : Interference filters, S : Leaf sample, Sl : Slit, H : Sample holder, P : Photocell, E₁ : Drycell for photocell, E₂ : Drycell for lamp, M : Meter.

Table 3. Example of measurement on various leaves by chlorophyllo-meter.

Name of plant	Deepness of green color	The other coloration	Reading
<i>Oryza sativa</i>	—	Maple (Dead)	0.02
	—	Yellow	-0.01
	+++	—	0.50
	+++	—	0.57
	?	Purple	0.55
<i>Euonymus japonica</i>	—	Yellow	-0.01
	—	〃	0.01
	—	〃	0.02
	+++	—	0.42
	+++	—	0.52
<i>Hordeum vulgare</i>	+	—	0.09
	++	—	0.20
	++	—	0.22
<i>Fragaria chiloensis</i>	—	Red	0.06
	++	—	0.24
<i>Brassica napus</i>	—	Reddish Orange	0.04
	+	—	0.15
	++	—	0.22
<i>Canna generalis</i>	—	Brownish (Dead)	0.07
	+++	—	0.43
	+++	—	0.77
<i>Podocarpus macrophylla</i>	+	Yellowish	0.09
	++	—	0.14
	+++	—	0.29
	+++	—	0.49

A trial product of the chlorophyllo-meter was tested on the relation between the reading expressed as the value of A670—A750 and the deepness of green color using various leaves. In all of the plants tested, a clear relationship was obtained in each plant, as shown in table 3.

A relationship between the reading and the chlorophyll content of unit leaf area was also studied on rice leaves, giving a very close correlationship between them as shown in figure 8. It is indicated that the difference attenuance expressed as A670—A750 in each leaf shows quite accurately the

Fig. 8. Correlation between the reading of chlorophyllometer and the chlorophyll content per unit area of rice leaves.

chlorophyll content in the leaf per unit area.

In this method, the reading keeps a linear relation with the chlorophyll content, unlike the reflectance method¹⁾, even the content is very high. The reading is not affected by the glossiness of leaf surface. Accordingly, this method can be applied to many kinds of plant leaf for estimating their deepness of green color and chlorophyll content per unit leaf area, regardless of whether the upper surface or the reverse surface of the leaf is held facing to the light source. From above results, it may be concluded that this method is quite suitable for estimating the deepness of green color and the chlorophyll content per unit area of the leaves.

SUMMARY

In order to find out a quick and simple method for estimating the deepness of green color and the chlorophyll content of crop leaves, a study was carried out to get the following results.

(1) The attenuance spectrum, which is a term given for the absorbance spectrum of non-transparent

substances, was determined by the spectrophotometer using various living leaves. A peak attenuance band was observed at the wave length of about 670 mμ and a minimum band in the range beyond 750 mμ in all of the leaves used in the experiment.

(2) The difference attenuance, which is expressed as the balance of attenuance at 670 mμ minus that at 750 mμ (A₆₇₀—A₇₅₀), indicated a close relationship between the deepness of green color and the chlorophyll content in the leaves.

(3) A chlorophyllometer was produced for a trial on the basis of the principle of difference attenuance, and was tested. The reading of the meter coincided well with the deepness of green color of leaves and a high correlation was obtained between the reading and the chlorophyll content per unit area of leaves.

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[和文摘要]

作物生葉の緑色程度ならびに葉緑素含量の測定法とその応用に関する研究

1. 生葉の緑色程度ならびに葉緑素含量の測定原理について

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作物葉の緑色程度ならびに葉緑素含量を生葉のままで簡易、迅速に測定する方法を明らかにするために研究を行ない、次のような結果を得た。

1. 生葉の attenuance スペクトル（透明物質における吸収スペクトルに相当する言葉で、非透明物質に対して用いられる）を測定したところ、用いたすべての植物において葉緑素に基づく極大値は 670 mμ またはその近くにあり、750 mμ 以上では葉の色素に無関係な基線と呼ばれる一定の最低値を示すことがわかった（図 2, 3,

4, 5).

2. 同一葉における difference attenuance すなわち $670\text{ m}\mu$ と $750\text{ m}\mu$ における attenuance の差 ($A_{670} - A_{750}$) を求めることによつて, 単位葉面積当りの葉緑素含量を生葉のままで測定しようと考えた. また, この difference attenuance の値は葉の緑色程度と密接な平行関係を示すことが用いたすべての植物で認められた (表 2, 図 4, 5).

3. difference attenuance を直接, 簡易に測定するために携帯用葉緑素計を試作し試験したところ, 用いたすべての植物において葉の緑色程度と示度との間に密接な平行関係がみられ (表 3), また, 水稻葉で単位葉面積当りの葉緑素含量と示度との間にはきわめて高い相関々係のあることが認められた (図 8).