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Comparative Study on Several Determination Methods of Chlorophyll Content in Plants

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Abstract. Chlorophyll was determined in the N,N-dimethylformamide (DMF) and the mixture solution ($V_{alcohol}/V_{acetone}$=1:1) respectively, and the extracted effects were compared. The results showed that the extracted effects of chlorophyll content in the mixture solution method is better than that of DMF method when the chlorophyll content was low, while DMF method is better when the chlorophyll content was high. DMF extraction method with long extraction time was better to the coniferous forest vegetation and *Ginkgo biloba* Linn., because the plant's well-developed cuticle on the blade surface could hinder the dissolution of chlorophyll in the extract in a short time. The SPAD values were measured when leaves were determined by above two methods meanwhile, and were linearly fitted with the actual chlorophyll content values to obtain the linear correction equation. It found that SPAD values had a good linear relationship with chlorophyll content (with mg/dm^2 fresh weight, R^2 >0.96). Linearity range of SPAD value is 25~60 in DMF method, and 26~50 in the mixture solution method.

1. Introduction

Chlorophyll is an important photosynthetic pigment in plants. The chlorophyll content of plants can reflect the photosynthetic energy efficiency, so it can be used as a basic physiological index to study plant growth and development.[1]

There are many methods for the determination of chlorophyll content of plants by spectrophotometer [2]. Among them, Arnon method [3,4], DMF (N,N-dimethylformamide) method[5,6] and mixture solution ($V_{alcohol}/V_{acetone}$=1:1) method[7] were widely used. Arnon method was put forward by Mackinney[3], then was explained and deduced by Arnon[4]. DMF method was put forword by Moran [5,6]. The mixture solution method was put forward by Xianzheng Zhang in 1985[7]. Arnon method of chlorophyll extraction was cumbersome steps and heavy workload, and influencing accuracy easily by unstable the extraction [8]. DMF method was simple and efficient because DMF has the same effect of inhibiting chlorophyll enzyme activity as acetone but poisonous. The mixture solution method was simple, too. There are many improvements and comparative studies on this method at present [1,8,9].

In the 1960s, Japan began the study in nondestructive chlorophyll analyzer [10]. The nondestructive chlorophyll analyzer can measure chlorophyll content without damage to plants, which makes up for the shortcoming that the above methods can only measure chlorophyll in vitro. Therefore this type of analyzer developed rapidly, and the research about on nondestructive chlorophyll determination technology and the correlation between the results of nondestructive chlorophyll analyzer and actual chlorophyll content appeared extensively [10-14]. At present, SPAD value which represented nondestructive testing data was widely used.

In this study, DMF method and the mixture solution method ($V_{alcohol}/V_{acetone}$=1:1) were selected to compare the extracting effection of chlorophyll content used seven plant species. SPAD values were determined at the same time to establish correction equation in order to provide reference for rapid extraction and determination of chlorophyll in batch samples.

2. Materials and Methods

2.1. Plant Material

Seven species of plants were selected as plant materials for this study, including *Fraxinus chinensis* Roxb., *Ginkgo biloba* Linn., *Magnolia denudata* Desr., *Platycladus orientalis* (L.) Franco, *Pinus tabulaeformis*, maize and soybean. The experimental plants of *P. alba* var. were two years-old seedlings, maize and soybean were seedling which potted in early April, and the other experimental plants were adult trees.

2.2. Chlorophyll Content Measurements

In April and June, fresh leaves of *Fraxinus chinensis* Roxb., *Ginkgo biloba* Linn., *Magnolia denudata* Desr. were picked for chlorophyll content determination in weight (mg/g) and in area (mg/dm^2).

In May, fresh leaves of *Platycladus orientalis*(L.) Franco, *Pinus tabulaeformis*, maize and soybean were picked for chlorophyll content determination in weight (mg/g) and in area (mg/dm^2).

The tested leaves were washed with steam water and the surface water of leaves was absorbed with filter paper.

The leaves of *Fraxinus chinensis* Roxb., *Ginkgo biloba* Linn. (in June), *Magnolia denudata* Desr. were sampled with a hole punch (disc, 1cm in diameter) on one side of the main vein, then 4 pieces in each group (area was π) were added DMF. The other side of the main vein of leaves was sampled as above (disc, 1cm in diameter, area is π) and added the mixture solution ($V_{alcohol}/V_{acetone}$=1:1).

Four leaves of maize and soybean were picked respectively. Two discs were sampled with a hole punch (1 cm in diameter), each one disc was picked to mix in a group with four leaves and tested. Because the leaves of *Ginkgo biloba* Linn. (in April) were small, the chlorophyll content is determined in weight (mg/g). The leaves of *Platycladus orientalis*(L.) Franco and *Pinus tabulaeformis* were needles, therefore the chlorophyll content is determined in weight (mg/g).

Samples were cut into small pieces about 1 mm, and then put into test tube.

2.2.1. DMF method. The extract (DMF, 5 ml) was added into each test tube, and was kept away from light for 24 hours at 4 °C, shaking occasionally. The sample supernatant was measured at 647nm and 664.5nm by 722 visible spectrophotometer. The chlorophyll content was calculated by formula (1-3)[5,6].

$$Chla=12.70A664.5 -2.79A647 \quad (1)$$

$$Chlb=20.70A647-4.62A664.5 \quad (2)$$

$$Total\ Chl=17.90A647 +8.08A664.5 \quad (3)$$

2.2.2. The mixture solution method. The absorption spectra of chlorophyll solution extracted by the mixture solution method were consistent with those of chlorophyll extraction solution by Arnon method[4,7]. Therefore, the formula of Arnon method was used to calculate the chlorophyll content here.

The extract (the mixture solution, $V_{alcohol}/V_{acetone}$=1:1, 10ml) was added into each test tube, and was kept away from light for 2 hours at 50°C [7,8]. The sample supernatant was measured at 645nm and 663nm by 722 visible spectrophotometer. The chlorophyll content was calculated by formula (4-6).

$$Chla=12.70A663-2.69A645 \quad (4)$$

$$Chlb=22.90A645-4.68A663 \quad (5)$$

$$Total\ Chl=20.20A645+8.02A663 \quad (6)$$

2.2.3. SPAD-502 plus analyzer. The SPAD values were measured by SPAD-502 plus (Konica Minolta) at the same time when leaves were sampled as the above method.

3. Result

3.1. Comparison of Two Extraction Methods

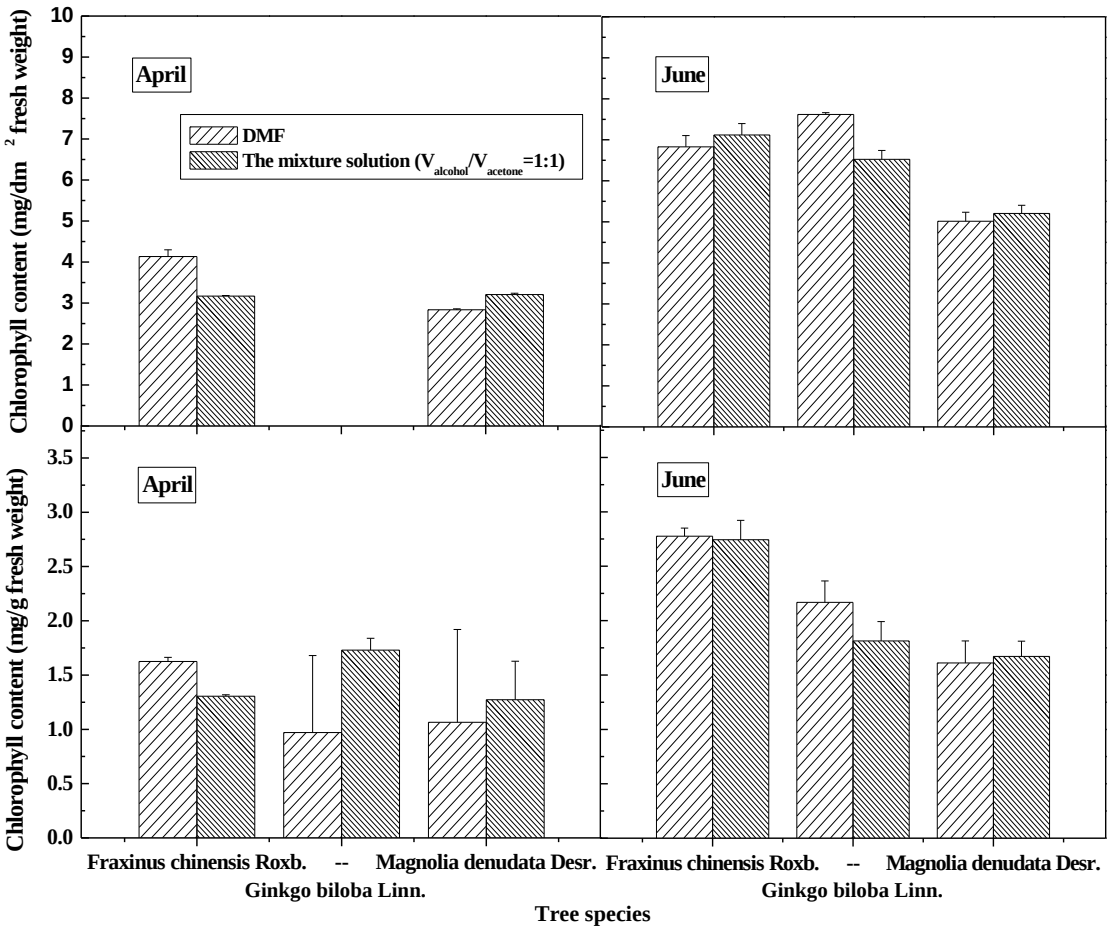

Figure 1. Chlorophyll content (mg/g fresh weight, mg/dm^2 fresh weight) of *Fraxinus chinensis* Roxb., *Ginkgo biloba* Linn. and *Magnolia denudata* Desr. with DMF method and the mixture solution method ($V_{alcohol}/V_{acetone}$=1:1) in April and June.

Figure 1 and figure 2 show the results of chlorophyll content determination of seven plants by two different extraction methods in different months. The mixture solution method has higher measured values in *Magnolia denudata* Desr., maize and soybean. The DMF method has higher measured values in *Platycladus orientalis*(L.) Franco, *Pinus tabulaeformis.* The measurements are similar with two extraction methods in *Fraxinus chinensis* Roxb..The mixture solution method has higher measured values in *Ginkgo biloba* Linn. in April, but it is higher in June with the DMF method.

3.2. Changeing of Chlorophyll Content

Fig. 1 shows the changes of chlorophyll content in April and June in three plants (*Fraxinus chinensis* Roxb., *Ginkgo biloba* Linn., *Magnolia denudata* Desr.). In April, leaves of plants are mainly tender leaves which is with light leaf color and low chlorophyll content. In June, the leaves are mostly mature leaves when plants grow vigorously. The color of leaves in June is greener than that in April, therefore all the three plants show the measured values are higher in June than in April.

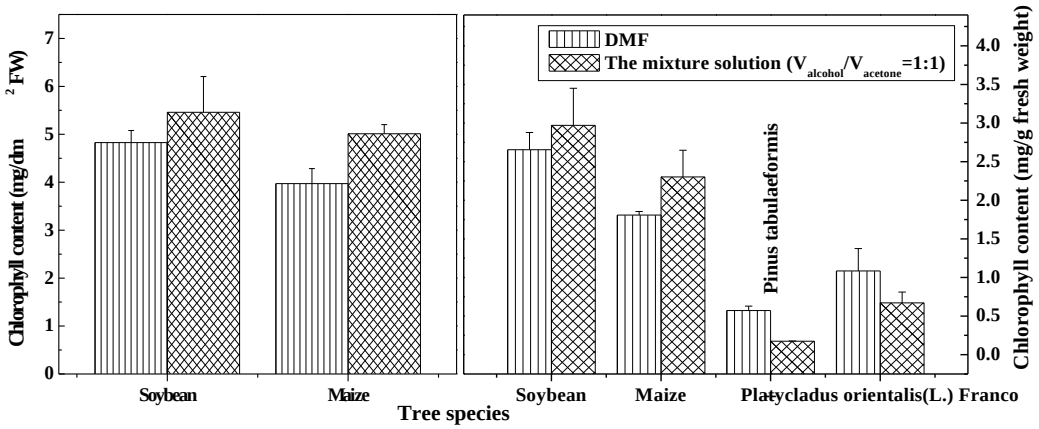

Figure 2. Chlorophyll content (mg/g fresh weight, mg/dm^2 fresh weight) of soybean, maize, *Pinus tabulaeformis* and *Platycladus orientalis* (L.) Franco, with DMF method and the mixture solution method ($V_{alcohol}/V_{acetone}$=1:1) in May.

3.3. SPAD Linear Correction Equation

The SPAD values were measured when leaves were determined by DMF method and the mixture solution method meanwhile. The SPAD values were linearly fitted with the actual chlorophyll content values to obtain the linear correction equation. The independent variable was SPAD, and the dependent variable was chlorophyll content. Linear correction equation and R^2 are shown in Table 1.

Table 1. SPAD linear correction equation

Determined methods	Linearity range of SPAD value	Unit of Chlorophyll content	Linear correction equation	R^2
DMF Method	25~60	mg/dm^2 FW	y = 0.186x - 1.933	0.9859
		mg/g FW	y = 0.1305x - 2.4186	0.7252
The mixture solution method ($V_{alcohol}/V_{acetone}$=1:1)	26~50	mg/dm^2 FW	y= 0.1879x - 1.8667	0.9602
		mg/g FW	y= 0.1219 - 1.9835	0.8797
	26~60	mg/dm^2 FW	y= 0.1787x - 1.5844	0.984
		mg/g FW	y= 0.0339x + 0.7437	0.2221

Table 1 shows that the linear relationship between chlorophyll content expressed in area and SPAD value is better. Linearity range of SPAD value is 25~60 in DMF method. Linearity range of SPAD value is 26~50 in the mixture solution method. When the measurement exceeds 50, the R^2 of linear correction equation appears serious deviation (y = 0.0339x + 0.7437, R^2 = 0.2221, mg/g FW).

4. Discussion

Chlorophyll is a kind of magnesium porphyrins, which is unstable. It can be decomposed in the presence of light, acid, alkali, oxygen and oxidant etc.

DMF can inhibit the activity of chlorophyll enzymes effectively, and the extraction method is easy to operate. Therefore DMF method is widely used at present. However, DMF method has a long extraction time (24h) and DMF is high toxicity. Therefore, the mixture solution method (acetone/ethanol) was proposed by domestic scholars and several improvements about the method were made. The mixture solution method is easy to operate and is widely used at present too, because of the short extraction time (2h) and fast determination. But the mixture solution method needs higher temperature and needs to avoid light when extracting, while the improper operation is easy to affect the extracted effects of the chlorophyll content.

Three plant species were selected to compare the above two extraction methods in April and June. The results show that the extracted effects of chlorophyll content in the mixture solution method is better than that of DMF method when the chlorophyll content was low in April, while DMF method is

better when the chlorophyll content was high in June. That may be related to the interannual variation of chlorophyll content and the extracted time of the two methods. The extracted time of the mixture solution method (2h) is shorter than that of DMF method (24 hours). When the chlorophyll content is low, the extraction effect of the two methods is similar. When the chlorophyll content is high, the short extracted time would lead to the incomplete extraction of chlorophyll. Therefore, it is suggested to use DMF method when the plant species with high level of chlorophyll, and to use the less area or weight of leaves with the mixture solution method.

The extracted effect of the DMF method is obviously better than with the mixture solution method in *Platycladus orientalis*(L.) Franco, *Pinus tabulaeformis and Ginkgo biloba* Linn. (in June). *Platycladus orientalis*(L.) Franco and *Pinus tabulaeformis* are coniferous forest vegetation which has well-developed cuticle on the blade surface[15]. *Ginkgo biloba* Linn. is deciduous tree specie, while its leaf is similar to that of *Platycladus orientalis* (L.) Franco and *Pinus tabulaeformis* with well-developed cuticle on the blade surface. Insoluble proteins in cuticle may hinder the dissolution of chlorophyll in the extract in a short time. Therefore, DMF method is recommended for the extraction of chlorophyll from leaves with thicker cuticle. DMF method could be more completely destroyed the protection of cuticle to leaves with a long extracted time.

At present, the main methods for determining chlorophyll content are spectrophotometer method and the nondestructive chlorophyll analyzer. SPAD value is relative chlorophyll content which is measured by the nondestructive chlorophyll analyzer, therefore the spectrophotometer method that can determine absolute chlorophyll content is more widely used [1]. The spectrophotometer method has a variety of determination methods because of using the different solvents. Most of the determination methods have heavy workload, complicated steps, long extracted time or strict extraction conditions. Therefore, improper operation can lead to errors easily, and the determination methods are not suitable for the extraction and determination of a large number of samples [8]. SPAD-502 plus chlorophyll meter could be used to measure the chlorophyll content rapidly and in real time. After recording the SPAD values, the actual chlorophyll content could be obtained through the linear correction equation. Therefore, SPAD value and chlorophyll content could be carried out simultaneously at the beginning of the experiment in order to obtain the linear correction equation and the linearity range of SPAD value. That could save time for chlorophyll content determination for the subsequent experiment.

A number of studies have used the SPAD value to express chlorophyll content directly at present [16-20]. However, table 1 show that the SPAD values which could indicate the chlorophyll content accurately has a certain linearity range. If the SPAD values exceed the linearity range, the quantitative conversion and analysis of chlorophyll content could not be carried out. Therefore, in order to obtain more reliable data, it is recommended to obtain the the linear correction equation and the linearity range of SPAD value first according to the different extraction methods of chlorophyll content with the characteristics of plant leaves. At the same time, the different units of chlorophyll content need to be considered when the linear correction equation and the linearity range of SPAD value are obtained.

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6. References

[1] Fen X, Wei L, Hong-Yan L, Ling-Yun Z, Ding D, Zhen Z 2016 Comparisonon methods of chlorophyll extraction in camellia sinensis. *Journal of Tea Communication* 43(04):37-40
[2] Zheng-Shu S and Xian-Zheng Z 1989 Comparison of several methods for determining plant chlorophyll content *Plant Physiology Communications* 5:77-78
[3] Mackinney G 1941 Absorption of Light by Chlorophyll Solution *J. B iol. Chem.* 140: 315-322
[4] Arnon D I 1949 Copper Enzymes in Isolated Chloroplasts Phenoloxidases in Beta Vulgaris *Plant Physiol.* 24:1-15
[5] Rami M and Dan P 1980 Chlorophyll Determination in Intact Tissues Using N,N-Dimethylformamide *Plant Physiol.* 65:478-479

[6] Rami M 1982 Formulae for Determination of Chlorophyllous Pigments Extracted with N,N-Dimethylformamide *Plant Physiol.* 69: 1376-1381
[7] Xian-Zheng Z 1986 Determination of Plant Chlorophyll Content by Acetone-Ethanol Mixture Method *Liaoning Agricultural Science* 3:26-28
[8] De-Xiao L, Wan-Wei H, Hai-Yan Y 2006 Fast-soaking methods of chlorophyll from maize leaf *Jour. of Northwest Sci-Tech Univ. of Agri. and For. (Nat. Sci. Ed.)* 34(11):65-67
[9] Fa-Shui H, Zheng-Gui W, Gui-Wen Z 2001 Soaking and synergistic extracting reaction of chlorophyll from spinach *Chinese Journal of Applied Chemistry* 18(7):532-535
[10] Zhang-Qing Y, Jian-Dong H, Tie-Cheng D, Shun W, Jian-Zhong W, Wei-Bo S, Yu-Min Z 2019 Research Progress of Nondestructive Diagnostic Technique of Chlorophyll in Plants *Chinese Agricultural Science Bulletin* 35(07):139-144
[11] Uddling J, Gelang-Alfredsson J, Piikki K, Pleijel H 2007 Evaluating the relationship between leaf chlorophyll concentration and SPAD-502chlorophyll meter readings *Photosynthesis research* 91(1):37-46
[12] Cate T.M. Perkins T.D. 2003 Chlorophyll content monitoring in sugar maple (acer saccharum) *Tree Physiology* 23(15):1077-1079
[13] Xiao-Sheng Z, Yue-Qin Z, Lei P, Chang-Jun J, Ye-Yun L 2012 Estimate of leaf chlorophyll and nitrogen content in camellia sinensis by ccm-200 *Journal of Anhui Agricultural University* 39(01):150-153
[14] Pingzeng L, Guansheng Z, Zhenmin D, Wenshan W, Yucun W, Qingkuan M, Shusheng B 2012 Development of a low-cost crop chlorophyll detector *Computer Measurement & Control* 20(4):1155-1158
[15] Chen-Hai H and Jian L 1993 Analysis of the Anatomical Structure of Tree Leaves from Western Liaoning *Journal of Shenyang Agricultural University* 24(4):269-273
[16] Guo-Xiang S,Xiao-Chan W, Ye S, Yong-Qian D, Wei L 2019 Measurement Method Based on Multispectral Three-Dimensional Imaging for the Chlorophyll Contents of Greenhouse Tomato Plants *Sensors (Basel, Switzerland)* 19(15)
[17] Li-Ping R, Jia-Qi Z, Dong-Hui C, Ji-Sheng L, Yan Z, Yu-Fu G 2019 Effects of simulated acid rain on physiology and chlorophyll fluorescence parameters in Acer truncatum seedlings *Non-wood Forest Research* 3:44-51
[18] Rui L,Jun-Hui C,Yong-Lin Q,Ming-Shou F 2019 Possibility of using a SPAD chlorophyll meter to establish a normalized threshold index of nitrogen status in different potato cultivars *Journal of Plant Nutrition* 42(8)
[19] Swiader J M and Moore A 2002 Spad-chlorophyll response to nitrogen fertilization and evaluation of nitrogen status in dryland and irrigated pumpkins *Journal of Plant Nutrition* 25(5):1089-1100
[20] Yu-Jie H, Lin-Nan W, Jian-Rong F, Baimaduoji, Fu-Xia W, Xin-Min R 2016 Effects of NaCl stress on photosynthetic characteristics of leaves of the two grape cultivars *Xinjiang Agricultural Sciences* 53(10):1794-1800