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Methods for determining leaf chlorophyll content of rice: A reappraisal

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Estimation and determination of chlorophylls a and b from rice leaves by different methods have been evaluated. A simple and easy incubation method dispensing grinding and centrifugation procedures is described. The recovery of chlorophyll pigments by incubating rice leaf tissues in 80% buffered acetone at 4°C gives higher yields of pigments compared to that of incubation at 36°C. The use of acetone to extract chlorophyll by incubation is found to be superior to other methods in extraction of pigments.

Many methods are available to extract and quantify chlorophyll from plant tissues using wide range of organic solvent[1]. But there are drawbacks associated with each of these methods in extracting chlorophyll from leaves[2]. Extraction with organic solvents necessitates a number of procedural steps which inevitably results in some loss of pigments. Grinding of leaf tissues does not result in complete extraction of pigments all the time. Methods which involve grinding and centrifugation of tissues require a relatively high volume of solvents leading to the lowering of the concentrations of pigments in the final volume. Hence, extraction is not achieved completely when working with etiolated plants where concentration of chlorophyll is low and also when the material available for sampling is limited. Recent methods using solvents like dimethyl formamide (DMF)[3] and dimethyl sulfoxide (DMSO)[4] circumvent the difficulties arising due to the maceration and centrifugation. DMF and DMSO are toxic to human as they are easily absorbed by the skin[5] however acetone is less toxic with a tolerance of up to 1000 ppm. Extraction of chlorophyll from rice leaves poses some more problems. Rice plant has more silica content[6] than any other plant as a result, with the advancement of plant growth, the leaf tissues become difficult for maceration. Consequently the simplication of the procedures are al ways sought which enable complete extraction and spectrophotometric determination of chlorophyll in rice leaves.

Here we report a simplified procedure for improved extraction of chlorophyll from rice leaves dispensing maceration and centrifugation by incubating the leaf tissues in 80% buffered acetone at 4°C. During this investigation we also performed a comparative analysis of the commonly used methods for extraction and spectrophotometric determination of chlorophyll from rice leaves.

Materials and Methods

Leaf samples—Rice leaves of the cultivars, viz. IR 36, Ratna, Swarnaprabha, FR 13 A and CR 1009 were excised from the pot grown plants and were brought to the laboratory in polythene bags, lined with moist filter paper inside. The leaves were washed thoroughly in running cold water for 5 min and then cut into small discs of known area with the help of a disk cutter. The pattern of leaf cutting ensured a proper representation of the total leaf. A sample of leaf discs was weighed immediately and was preserved for dry weight measurement. Another similar sub sample of these leaf discs was further cut into pieces (of approximately 0.2-0.4 cm^2) and then used for extraction and estimation of chlorophyll. Care was taken to avoid the excessive loss of moisture from leaf discs by preserving them in distilled water. Five replicates were prepared from five different plants of the same variety.

Chemicals and reagents—Chemicals used in this study were of analar grade. To avoid the decomposition of the chlorophyll pigments, all glassware and solvents were made from from acids, bases and reducing or oxidising substances[7]. Residues of acids on glassware were washed off with a concentrated solution of sodium phosphate[8].

Distilled water from an all glass apparatus, with no addition of potassium permanganate, was used. All procedures were performed under diffused light to eliminate the exposure of leaf materials to direct, bright or sun light[9].

Estimation of chlorophyll content by acetone incubation method—Rice leaf tissues (100 mg) were placed in a graduated tube containing 25 ml of 80% buffered acetone (80 ml of acetone made up to 100 ml with 20 ml of 2.5 m*M* sodium phosphate buffer, *p*H 7.8) and the chlolrophyll was extracted without grinding and centrifugation, by incubating the leaf tissues into the solvent in a dark place. The contents of the tubes were shaken occasionally to accelerate the pigments extraction. At the desired period of incubation the extract liquid was filtered through glass wool to remove leaf pieces and transferred to another graduated tube. Then the extract liquid was made up to a total volume of 25 ml with 80% buffered acetone. After checking the tubidity of the extracts at 750 nm, the chlorophyll content was spectrophotometrically analysed, in a dual beam recording UV visible spectrophotometer (Beckman UV-VIS 35, USA) using 3 ml sealed quartz-glass cuvettes with a path length of 1 cm. The chlorophyll content was calculated following the equations proposed by Barnes *et al.*[10]. The chlorophyll content was measured at three hourly intervals till there was a decline in the total content of chlorophyll at two different incubation temperatures of 4°±2° and 36°±2°C. at three hourly intervals.

Comparative analysis—For the purpose of comparison, chlorophyll extracts were also prepared by using various solvents like 80% acetone[11], 96% ethanol[12], methanol[13] and dimethyl sulfoxide[4]. Absorbency of the extracts was then read at wavelength correspo nding to each solvent. Chlorophyll concentration of the extract liquids prepared from 80% acetone and DMSO were calculated using the equations proposed by Barnes *et al.*[10]. While the concentrations of the extract liquids prepared in methanol and ethanol were calculated according to the proposed equations in the respective methods, the phaeophytinization (O.D. 435/O.D. 415) an estimate of the degree of chlorophyll degradation in pigment extracts was quantified for all the methods.

Results and Discussion

Chlorophyll extraction at two different temperatures (4 and 36°C) showed a maximum around 27 hr. The extraction and recovery of total chlorophyll at 4°C was about 10% more than that of the extraction at 36°C. The Pqa of the extract liquid at 4°C was about 6% more than that of the extract liquid at 36°C.

Comparison of different methods for extraction of chlorophyll—Data on different solvents and methods for extraction of chlorophyll from the rice cultivars given in Table 1 indicated a significant ($P<0.05$) effect s of solvents and methods on the chlorophyll content and Pqa. Among the methods compared, the acetone incubation method was superior to others, except in cv. Ratna where the recovery of chlorophyll was not significantly different between DMSO and acetone incubation methods.

Acetone incubation method has improved chlorophyll recovery by 24% more than the conventional Arnon's method. Chlorophyll extraction by grinding leaves in solvents and then centrifugation of the extracts resulted in incomplete recovery of chlorophyll. Following centrifugation some green colour in the tissues was also noted in the conventional Arnon's method. The acetone incubation and DMSO methods showed higher values of Pqa in all the cultivars indicating a marked reduction in chlorophyll degradation. But the recovery of chlorophyll was 7% more in the acetone incubation method than in the DMSO method.

When acetone incubation method was tested against DMSO method the chlorophyll a and b were extracted more than the DMSO method by about 9% and 11% respectively. The phaeophytinization quotient was comparable in both the treatments indicating the competency of incubation method with DMSO method.

Most of the currently available chlorophyll extraction procedures involve many steps which either dilute or lead to the loss of pigments. Though DMSO method avoids these losses, the turbidity of the extract liquid when matured leaves are used pose problems. The simplified procedure followed in the present study "Acetone incubation method" is superior to many others in extracting chlorophyll

Table 1—Chlorophyll content[1] and phaeophytinization quotient[2] of rice leaf extracts by different methods of extraction and determination

Treatment	IR 36		Ratna		Cultivar FR 13A		Swarnaprabha		IR 64	
	Chl a+b	PQa	Chl a+b	PQa	Chl a+b	PQa	Chl a+b	PQa	Chl a+b	PQa
Acetone 80% (Maceration)	1.872^{c}	1.175^{cb}	2.906^{c}	1.285^{c}	3.112^{c}	1.305^{bc}	2.643^{c}	1.234^{c}	2.401^{c}	1.251^{c}
Methanol	1.453^{a}	1.066^{a}	2.379^{a}	1.227^{a}	2.486^{a}	1.124^{a}	2.139^{4}	1.119^{a}	2.004^{a}	1.179^{a}
Ethanol	1.741^{b}	1.075^{b}	2.494^{b}	1.175^{b}	2.604^{b}	1.192^{b}	2.256^{b}	1.129^{b}	2.145^{b}	1.122^{b}
DMSO	2.229^{d}	1.202^{d}	3.723^{d}	1.314^{d}	3.978^{d}	1.335^{d}	3.392^{d}	1.262^{d}	2.775^{d}	1.283^{d}
Acetone 80% (incubation)	2.401^{e}	1.251^{d}	3.990^{d}	1.379^{e}	4.167^{e}	1.392^{e}	3.651^{e}	1.322^{e}	2.824^{e}	1.272^{e}

[1] Chlorophyll a+b in mg g^{-1} fresh weight

[2] Pqa: Phaeophytinization quotient (ratio of OD) 435 / 415)

Means followed by the same subscript within a column are not significantly different at $P < 0.05$ Duncan's Multiple Range test.

pigments from rice leaves. This method eliminates the procedural steps, both grinding and centrifugation and is as effective as DMSO method in the recovery of chlorophyll pigments. However the incubation method showed a complete extraction of chlorophyll pigments with less of turbidity.

The recovery of chlorophyll using extracts from solvents like methanol and etha nol with grinding of leaf tissues followed by centrifugation resulted in poor yield of pigments when compared to that of buffered 80% acetone using Arnon's method.

Incubation period required for mature rice leaves in 80% buffered acetone was 27 hr. The subsequent extraction neither yielded additional chlorophyll nor was the remaining plant material green, which clearly showed that the chlorophyll extraction was complete.

The acetone incubation method has improved the recovery of chlorophyll by about 25% when compared to the Arnon's method. This may be partially explained by the prevention of loss of chlorophyll pigment during the procedural steps of extraction like grinding and centrifugation[9]. In addition to the above reasons, in the conventional maceration methods, chlorophyll may also be left in the leaves due to incomplete grinding.

Another disadvantage of the conventional method is that the absorbency of extracts must be read immediately following grinding and centrifugation; it is not possible to store extracts and measure the absorbency at a latter date without marked chlorophyll degradation[14]. But the acetone incubation method eliminates this problem completely as the extracts prepared at 4°C in buffered 80% acetone showed less than 10% degradation of chlorophyll over a period of 45 hr.

In the current study, the degree of phaeophytinization of pigment extracts was assessed using the phaeophytinization quotient (Pqa) a reliable parameter for estimating degradation of chlorophyll a to its magnesium free derivative-phaeophytin a[15]. It was clearly established that the period of incubation in acetone at 4°C resulted in no significant degradation of chlorophyll a. Though there were no significant changes in the Pqa between the acetone incubation method and DMSO methods, the chlorophyll recovery was low in DMSO. This low chlorophyll recovery in DMSO might be due to degradation of pigments caused by boiling of extract liquids in DMSO.

When working with plant species other than rice, it is important to check the period of incubation to ensure complete extraction of chlorophyll from the leaves as the time period of incubation would vary with different leaf types[4]. It may be determined by noting visually when the leaf tissue fragments appeared clear.

The acetone incubation method offers certain advantages (I) It eliminates the requirement for grinding plant tissue and centrifuging extracts, (ii) it is convenient and simple to use, (iii) the solvent used is neither corrosive nor toxic to the skin, (iv) preparation of many number of replicates is feasible, (v) chlorophyll can be completely extracted

from leaf types which are difficult to grind and also when the chlorophyll concentrations in the tissues to be studied are extremely low.

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