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## Studies of Chlorophyll Content by Different Methods in Black Gram (*Vigna mungo* L.)

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**Abstract:** Extraction, estimation and determination of different pigments from black gram leaves by different methods have been evaluated. The main pigments are chlorophyll a, b and phaeophytin content are studied at different wave lengths. Several methods are used for this purpose but a simple and easy incubation method dispensing grinding and centrifugation procedures is described. The recovery of chlorophyll pigments by incubation method in which tender leaf tissue in 80% buffered acetone at 4°C give higher yield of pigments compared to other methods. The use of acetone to extract chlorophyll by incubation is found to be superior to methods in extraction of pigments.

**Key words:** Chlorophyll content, methods, black gram, phaeophytin

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### INTRODUCTION

Currently we are having several methods for estimation of chlorophyll. Some methods involve many procedural steps, which leads to dilute, or loss of pigments. Though DMSO method avoids these but Incubation method is most accurate with less of turbidity. Most of traditional methods of pigment analysis (e.g., High Performance Liquid Chromatography, HPLC) are not ideal to obtain long term data, Sims and Gamon (2002). The extraction of chlorophyll pigments from leaf tissue of black gram using solvent like methanol with grinding of leaf tissue followed by centrifugation resulted in poor yield of pigments when compared to the other methods (Leung, 1998).

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), Moran (1982) and Dimethyl Sulfoxide (DMSO), Hiscox and Israelstam (1979) 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 (Jacob *et al.*, 1971) however acetone is less toxic with a tolerance of up to 1000 ppm.

Here we report a simplified procedure for improved extraction of chlorophyll from black gram 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 spectrophotometric determination of chlorophyll from black gram leaves.

### MATERIALS AND METHODS

#### Leaf Samples

Leaves of Black gram cv. T9 from different concentrations of physical mutagen (10-50 kR) and chemical mutagen (0.01-0.05% SA) collected and were brought to Laboratory in polythene bags, lined

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with moist filter paper inside. Care was taken to avoid the excessive loss of moisture from leaf by preserving them in distilled water. Five replicates were prepared from each concentration of physical treatment and five from chemical treatment.

### **Chemicals and Reagents**

To avoid the decomposition of chlorophyll pigments all glassware and solvents were made free from acids, bases and reducing or oxidising substances. Residues of acids on glassware were washed off with a concentrated solution of sodium phosphate (Association of Official Agriculture Chemists, 1960). Distilled water from an all glass apparatus, with no addition of potassium permanganate, was used. All the procedure were performed under diffused light to eliminate the exposure of leaf materials to direct, bright or sun light.

### **Estimation of Chlorophyll Content by Acetone Incubation Method**

Leaf tissues of black gram cv. T9 (100 mg) was placed in a test tube containing 10 mL of 80% buffered acetone (80 mL of acetone made up to 100 mL with 20 mL of 2.5 mM sodium phosphate buffer, pH 7.8) and the test tube were placed under refrigeration. At the desired period of incubation the extract liquid was filtered through glass wool to remove leaf pieces and transferred to another graduated tube. After checking the turbidity of extract at 750 nm, the chlorophyll content was spectrophotometrically analysed, in a dual beam recording UV visible spectrophotometer.

### **Estimation of Chlorophyll Content by Arnon Method**

A known amount of black gram leaf tissue 100 mg was suspended in 10 mL of 80% acetone, mixed well and kept at 4°C overnight in dark. Supernatant was withdrawn after centrifugation (5000 rpm) and absorbance was recorded at 663 and 645 nm in Spectrophotometer. The amount of chlorophyll was calculated according to Arnon (1949).

### **Comparative Analysis**

For the purpose of comparison, chlorophyll extracts were also prepared by using various solvents like 80% acetone (Arnon, 1949), methanol (Mackinnay, 1941) and dimethyl sulfoxide (Hiscox and Isrealstam, 1979) Absorbency of extracts was then read at wavelength corresponding to each solvent. The phaeophytinization (O.D. 435/O.D.415) an estimate of degree of chlorophyll degradation in pigment extract was quantified for all the methods.

## **RESULTS AND DISCUSSION**

The over all data obtained by using different solvents and methods for extraction of chlorophyll from black gram leaf tissues are presented in Table 1, which indicates significant effect of solvents and methods on the chlorophyll content and Pqa. The study also shows that incubation method is superior to the other methods. Superiority of incubation method have also been reported by Krishnan *et al.* (1996).

The Extraction of chlorophyll by grinding and centrifugation gives incomplete recovery of chlorophyll. The acetone and DMSO method gives higher values of Pqa in all the treatments. But recovery of chlorophyll was 8% more in the acetone incubation method than in the DMSO method. Present finding are in agreement with the results of Markwell (2002).

When acetone incubation method was tested against DMSO method the chlorophyll a and b were extracted more than the DMSO method by about 7 to 10%, respectively. The phaeophytinization quotient was comparable in both the treatment indicating the competency of incubation method with DMSO method.

Table 1: Chlorophyll content<sup>1</sup> and phauphytinization quotient<sup>2</sup> of black gram leaf tissues extracted by different methods and determination

Gamma rays treated leaves										
Methods	10 kR		20 kR		30 kR		40 kR		50 kR	
	Chl a+b	PQa	Chl a+b	PQa	Chl a+b	PQa	Chl a+b	PQa	Chl a+b	Pqa
Acetone 80% (Incubation)	2.872	2.245	3.876	2.202	2.511	2.111	2.191	1.713	2.422	2.511
Arnon method	2.351	2.602	2.411	2.020	2.332	2.981	2.010	1.710	2.004	1.115
DMSO	2.411	2.612	2.651	2.613	3.351	1.981	2.171	1.715	2.351	1.234
Methanol	2.210	1.990	2.131	1.714	2.131	1.711	1.991	1.615	2.001	1.230
Sodium azide treated leaves										
Methods	0.01%		0.02%		0.03%		0.04%		0.05%	
	Chl a+b	PQa	Chl a+b	PQa	Chl a+b	PQa	Chl a+b	PQa	Chl a+b	Pqa
Acetone 80% (Incubation)	3.871	2.175	2.711	1.951	2.884	2.121	4.168	2.173	2.451	1.951
Arnon method	2.110	2.100	2.135	1.851	2.151	1.951	2.571	2.101	2.121	1.651
DMSO	2.523	2.165	2.655	1.911	2.755	1.792	2.952	2.215	2.225	1.732
Methanol	2.101	2.111	2.112	1.751	2.101	1.113	2.551	2.131	2.113	1.362

<sup>1</sup>Chlorophyll a + b in mg g<sup>-1</sup> fresh weight, <sup>2</sup>Pqa: Phauphytinization quotient (ratio of OD) 435/415, Means followed by the same subscript with in column are not significantly different at p<0.05 Duncan's Multiple Range test

Most of the currently available chlorophyll extraction procedures involve many steps which either dilute or lead to loss of pigments. Though DMSO method avoids these losses, the turbidity of the extract liquid when matured leaves are used pose problems. So simplified method in present study Acetone incubation method is superior to many others extracting chlorophyll pigments from black gram 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 by using the solvent like methanol with grinding of leaf tissue followed by centrifugation resulted in poor yield of pigments when compared to that of buffered 80% acetone using Arnon (1949) methods.

The acetone incubation method has improved the recovery of chlorophyll by about 23% when compared to Arnon (1949) methods. This may be partially explained by the prevention of loss of chlorophyll pigment during the procedural steps of extraction like grinding and centrifugation. In addition above reason, the conventional methods, chlorophyll may 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, Lightenthaler (1987). 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 h.

The recovery of chlorophyll from the plants other than black gram it is important to check the period of incubation to ensure complete extraction of chlorophyll from the leaf tissues as the time period of incubation would vary with different leaf type, Hiscox and Israelstam (1979). It can be determined by noting visually when the leaf tissue fragments appeared clear Krishnan *et al.* (1996) in rice have drawn similar conclusion.

In short acetone incubation method proved superior to the other methods of extraction of chlorophyll. Some advantages are as (a) It is simple in use (b) Solvent used in it is not toxic to skin (c) It is not too procedural as it eliminate requirement of grinding and centrifugation (d) Chlorophyll are completely extracted by this method.

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