Quantitative and Qualitative Determination of Chlorophylls in Mutants of Soybean

by

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Introduction

Chloroplasts are found in all higher green plants and are of great biological importance. This organelle contains the pigments and other molecules necessary for converting light energy to chemical energy. This conversion is made possible by the possession of a light trapping pigment, chlorophyll. The chloroplast also contains the molecules, enzymes, necessary for using this trapped energy to convert inorganic compounds (carbon dioxide and water) into simple carbohydrate molecules. These processes are collectively referred to as photosynthesis. Higher plants contain two types of chlorophyll, a and b. These pigments give the green color to plants; and although green plants contain other pigments, the carotenoids consisting of the yellow xanthophylls and the orange carotenes, these pigments are hidden by the green chlorophylls. Some cells or areas in leaves do not possess chloroplasts as evidenced by the variegated appearance of the leaves of some plants (coleus is an example). Genetic mutations are known that result in a plant producing less than normal chlorophyll concentration in each cell or no chlorophyll at all. Plants with no chlorophyll live only until they have exhausted the nutrient from the seed and then they die. The number of chloroplasts is relatively constant in the cells of each species of plant. In higher plants, there are about 20 to 40 chloroplasts per cell of the green tissue. When the number of chloroplasts is insufficient, chloroplasts divide and increase in number; if the number is excessive, degeneration of the chloroplasts reduces the number. On the basis of this information, one may hypothesize that a reduction in chlorophyll content of a plant is due to an insufficient amount of chlorophyll in each chloroplast rather than to a reduced number of chloroplasts in each cell. A certain mutant of soybean involves a change in the chlorophyll producing mechanism. The gene for this characteristic exhibits a lack of dominance. One homozygous condition results in a plant with dark green leaves; the other homozygous condition gives a plant with yellow leaves; and the heterozygous condition gives rise to a plant with yellow-green leaves. These plants serve nicely to investigate the relationship between genetic makeup and the amount and type of chlorophylls in the plant leaf.

Various methods may be employed to determine the amount and type of pigments in plant tissue. We will utilize two of these—spectrophotometry and paper chromatography. The selective absorption of light, of a specific wavelength, is one method of identifying and quantitating chemical substances. Most biological materials absorb light in the 200 to 680 nanometer range (1nm = 0.001μ). Either the wavelength (color) of light absorbed or the amount of light of a specific wavelength absorbed by a solution can be used for qualitative analysis or quantitative analysis respectively. The term photometer refers to a light meter or measuring device. If the wavelength of light can be selected over a wide range of the spectrum by the machine, the instrument becomes a spectrophotometer. In spectrophotometry, a beam of light is passed through a solution containing a light absorbing material and the amount of light transmission or the "reverse" (the amount of absorbance) is compared to the transmission or absorbance through a known solution. The known solution may be the solvent alone in which case it is called the reference solution or blank; or the known solution may be the solvent plus a known amount of the light absorbing material in which
case it serves as a standard.

Chromatography may also be used to separate and identify substances in a mixture. There are several types of chromatography including column, paper, thin layer, and gas. Depending on the type of chromatography, the two phases may be gas and liquid, gas and solid, liquid and liquid or liquid and solid. Paper chromatography is one of the most extensively used chromatographic techniques with paper acting as the solid or stationary phase and the solvent as the mobile phase. The substance to be chromatographed is placed as a small spot or line near one end of a sheet of filter paper. This end is then immersed in a solvent system which usually is composed of two or more miscible substances. In ascending chromatography, the solvent is placed at the bottom of the chamber and allowed to rise upward by capillary action. As the solvent flows past the sample, it carries individual components along with it at a characteristic rate dependent upon their solubility in the solvent. When the solvent front approaches the upper end of the paper, the paper is removed and dried. This is referred to as a single dimension chromatogram. One can produce a two dimensional chromatogram by running the material one way and then by turning the paper 90° and permitting the solvent (same one or a different solvent) to move up the paper a second time. One must use sample spots rather than lines if one wishes to use two dimensional chromatography. The substances, once separated, can be identified by their color, by examination under ultraviolet light, by spraying with a chemical which reacts with the substance to form a colored compound, or by matching the Rf value of the substance with the Rf value of a known substance. The distance traveled by each compound from the origin or base line relative to the solvent front is defined as the Rf.

\[ R_f = \frac{\text{distance from base line traveled by compound}}{\text{distance from base line traveled by solvent}} \]

Spectrophotometric Determination of Chlorophyll Content of the Leaves of the Three Genotypes of the Soybean Mutant.

Members of the class will extract chlorophylls from leaves of each genotype and determine the amount or concentration present. Obtain a 0.5gm sample of leaves from one genotype; remove the petioles before weighing. Other members of the class will be performing a similar exercise with leaves from the other two genotypes. Grind the leaves with a mortar and pestle in 10ml of 80% acetone. Be careful not to spill any of the acetone solution; keep a small piece of filter paper handy to absorb any acetone solution that spills or runs down the mortar when the solution is being transferred. Use a small paint brush to keep the leaf pulp in the acetone solution. Filter the solution through Whatman #1 filter paper into a 100 ml flask. This can be done by placing a cone of filter paper into a small funnel which has the tube inserted in the mouth of the flask. Add another 10ml of acetone to the remaining leaf pulp and repeat the procedure. Repeat grinding the sample and filtering the solution until all the green color has been removed from the leaf pulp. Now take the filter paper cone and the piece of filter paper used to absorb spills and place it in the mortar and extract the pigment. The object is to get as much of the pigment as possible into the 100ml flask. Pour the solution from the flask into a graduate cylinder and add enough 80% acetone to bring the total volume to 100 ml. Place about 5ml of this solution in a Spectronic 20 tube and cover the top with parafilm to prevent evaporation of the solution. Prepare another tube with plain 80% acetone and cover. Set the Spectronic 20 to 0 absorbance with the 80% acetone blank before determining the amount of absorbance of your solution; this must be done for each wavelength. Now determine the absorbance of your chlorophyll solution at 645nm and 663nm. The values obtained from the cc (yellow)
plant are due to pigments other than chlorophylls; therefore, they should be subtracted from the Cc and CC plant values at 645 nm and 663 nm. After obtaining the corrected absorbance at the two wavelengths and using the equation given below, determine the concentration of chlorophyll in your leaf sample.

\[
D = \frac{mg \text{ total chlorophyll solution}}{mg \text{ of original suspension}}
\]

\[
D = \frac{801 \text{ acetone solution}}{(grams \text{ suspension}) 1000}
\]

*This would be 100 ml if you followed instructions, but could be whatever volume you used.

Amount of chlorophyll in CC (dark green) plant is ___________. The amount in Cc (yellow-green) plant is ___________. After obtaining the values from the CC and Cc plants, determine the ratio of chlorophyll in the CC plant to the Cc plant. What is your conclusion about the effect of each gene on chlorophyll production?

Separation and Identification of Plant Pigments Utilizing Paper Chromatography

Obtain a sheet of Whatman #1 filter paper. Take precautions not to finger the body of the paper as oil from your fingers will influence the flow of the solvent. Lightly pencil a line across the width of the paper about 1 inch from the bottom edge. Using a capillary tube place a thin line of your acetone extract of the plant pigments along the penciled line. Allow this to dry and reapply the solution. Repeat until the line appears to have a significant amount of green pigment. Obtain another piece of paper and apply the solution in a series of spots about 1 inch apart. Place both sheets in the solvent jar along with sheets containing pigments from the leaves of the other two genotypes. The jar contains solution A (petroleum ether and benzene, 9:1). Allow the solution to move up the paper until it is about 2 inches from the top of the paper. Take the paper out, allow it to dry and then place it in solution B (petroleum ether and benzene, 2:1). Again allow the solvent to move to about 2 inches from the top of the paper. Remove the paper and draw a line to indicate the solvent front (the highest point reached by the migrating solvent). Make drawings to show the appearance of your chromatograms. Determine the Rf value for each pigment; place a pencil mark in the center of each pigment spot to use as the distance traveled by the compound. Carotene is the most soluble in the solvent and therefore will appear close to the solvent front. The xanthophylls should be separated into two yellowish pigments below the carotene. Chlorophyll a is blue-green in color and chlorophyll b is yellow-green in color. Compare the pigments from each genotype. How do they differ as to types and amounts of pigments?

Relate the differences to the results obtained in the previous experiment.

Which chromatogram (the one produced from the pigment line or from the pigment spots) is better for determining the different types of pigments? The different amounts of pigment? 

The objectives for this laboratory exercise are:

1. To determine the quantitative and qualitative differences in pigment content of soybean plants with different genotypes for chlorophyll content.
2. To become aware that plants contain pigments that are masked by the chlorophylls.
3. To learn how to use the Spectronic 20 to quantitate a substance.
4. To learn how to use paper chromatography to separate substances (pigments) from a mixture.

Materials and Methods

1. Soybean seeds may be obtained from Carolina Biological #17-8200. They provide planting instructions and estimate of time required for plants to reach a specific size.
2. The 80% acetone solution is made up on a volume to volume ratio with water.
3. The filter paper cone is constructed by folding a 9cm circular piece of Whatman #1 filter paper into four equal parts.
4. A Spectronic 20 with operating instructions should be provided. The unit should be equipped with the accessory red filter and red sensitive phototube.

5. Provide several Spectronic 20 tubes in a test tube rack with several small squares of parafilm.
6. A pan balance sensitive to 0.01 gram should be available.
7. A pair of forceps should be used for handling the filter paper to remove it from the funnel.
8. A small artist's paint brush is ideal for pushing the leaf mass into the acetone.
9. Glassware should include a 100ml graduate cylinder, 100ml flask, small funnel, and mortar and pestle.
10. Teams of 2 to 4 members can be used to extract the pigment from each genotype of the soybean plant.