Hill Reaction of Photosynthesis-Effects of selected herbicides

Introduction:

The light reactions of photosynthesis provide a mechanism to convert low energy electrons in water to a high energy state in the form of the powerful reductant, NAPDH. The process is also coupled with the synthesis of ATP from ADP and inorganic phosphate. The components of photosynthetic electron transport, which 1) oxidize water, 2) transport electrons, and 3) synthesize ATP, are intricately arranged in, or along the sides of, the thylakoid membranes inside chloroplasts.

Since the discovery in 1937 by Robert Hill that illuminated leaf extracts could reduce ferric ions to ferrous state with the concurrent release of oxygen, many artificial electron acceptors have been discovered. These “oxidants” are useful for studying the process of photosynthesis because purified thylakoid membranes usually contain none of the natural electron acceptors such as NADP+ and ferrodoxin. Many of the artificial electron acceptors accept electrons at different sites along the transport pathway; this has facilitated the study of the functions of each part of the photosynthetic apparatus.

Some artificial electron acceptors, such as Paraquat has also been adapted as herbicide because they are relatively specific for plants. Other compounds have been discovered which block electron flow at specific sites along the transport pathway. Some of these compounds are also used as herbicides such as the urea-based Diuron and triazine-based Velpar. Finally, a widely used herbicide-Roundup (Glyphosate) is effective against a wide range of plants. Roundup is known to blocks the activity of one or more enzymes in the shikimic acid pathway that is involved with some essential aromatic amino acid synthesis.

As pointed out above, isolated thylakoids do not contain much of their complement of electron acceptors; thus we will use the dye DPIP as an artificial electron acceptor in determining the effects of four herbicides on photosynthetic electron transport by isolated thylakoids.

Procedure:

A. Preparation of thylakoids membrane: (Procedure done in light until end of step 5, thereafter complete all steps in the dark). Your TA will help in isolating thylakoid membranes for the whole class using following protocols:

1. Remove the blade tissues of 25g of washed spinach leaves. Place the de-veined leaves in a chilled blender top and add 25 ml of cold grinding medium (0.35 M Sucrose and 0.01M NaCl in 20 mM Hepes buffer at 7.8 pH).
2. Homogenize the tissues for 10 seconds using the Grind setting. Mix the tissues with pipette tips. Homogenize again for 10 seconds.

3. Filter homogenate through a filter into a beaker sitting on ice.
4. Divide filtrate evenly in two 50 ml tubes and centrifuge the tubes at ~ 5000 rpm for 10 minutes.
LOWER ROOM LIGHT LEVEL BEFORE REMOVING THE TUBES FROM CENTRIFUGE MACHINE

5. Carefully pour off supernatant and discard it. Pipette 5 mls of cold re-suspension buffer (20 mM HEPES, pH 7.8 with 0.01 M NaCl). Combine into one tube and use additional 5 mls of re-suspension buffer to rinse the empty tube. Combine the rinsed buffer to the thylakoid containing tube (altogether 15 ml) and carefully resuspend the precipitated thylakoid membrane.

6. Wrap the thylakoid preps in foil and keep on ice until ready to use.

7. When ready, your TA will aliquot 3 ml of thylakoid membrane prep to each table. Keep the prep in dark (Foil covered tubes)

8. Put 1 ml of thylakoid membrane prep in a glass test-tube and place in a boiling water bath for 10 minutes. Thereafter, wrap the tube in foil and place on ice.

B. Light-mediated electron transport by isolated thylakoids

1. Except for thylakoid prep, prepare tubes (LABEL THEM FIRST) by adding the solutions indicated in the summary table

2. Set wavelength to 610 nm and blank with (room temperature) resuspension buffer).

3. DO ONE TUBE AT A TIME. Begin each treatment's reaction with these steps:
a. Mark a point 12 inches from the provided light source.
b. mix thylakoid prep evenly by tapping the tube and add 0.1 ml of thylakoid membrane prep to the tube you are working with. Mix them by inverting the tubes several times.
c. TAKE READING IN THE SPECTROPHOTOMETER IMMEDIATELY. Numbers will be fluctuating so take the first number that comes up after inserting the tube and closing the chamber lid.

4. Remove the tube from the spectrophotometer and place it in front of a light source at a distance of 12 inches.

5. After 30 seconds, quickly invert the tube contents and then place it in the spectrophotometer and determine the decrease in absorbance- record it.

6. Repeat steps 4 and 5 until absorbance changes level off or become essentially zero.
7. Repeat steps 3 through 6 with each of the tubes (with light source off during preparation of each treatment). For dark control, place the tube in a drawer for 30 seconds between readings.

	TREATMENT							
(ml)	1*	1*	2	3	4	5	6	7
Resuspension buffer	3.75	3.75	3.60	3.60	3.60	3.60	3.75	3.75
0.1% DPIP (in ethanol) (Made Fresh)	0.15	0.15	0.15	0.15	015	015	0.15	0.15
0.2 mM Diuron (DCMU)	-	-	0.15	-	-	-	-	-
0.2 mM Paraquat	-	-	-	0.15	-	-	-	-
0.2 mM Velpar	-	-	-	-	0.15	-	-	-
0.2 mM Roundup (Glyphosate)	-	-	-	-	-	0.15	-	-
Light	yes	yes	yes	yes	yes	yes	no	yes
Dark	no	no	no	no	no	no	yes	no
Thylakoid Membrane prep	0.1	0.1	0.1	0.1	0.1	01	0.1	0.1 (boiled)

* duplicates

All of the reagents should be at room temperature except the thylakoid prep.

DATA SUMMARY AND CONCLUSIONS:

1. Prepare a single graph containing the data points for each treatment and properly label each line so generated (like Diuron rather than #2 tube)
2. For each plotted curve, determine the portion of the curve which attained linearity for 1.5 to 4 minutes. Determine the absolute absorbance change for this linear period and the

actual time intervals in minutes. Calculate the rate of Hill Reaction for the respective curves as absorbance change/interval linear time as follows

$$\text{Rate} = \frac{\text{Linear portion change in 610 absorbance (initial-final)}}{\text{Time in linear portion (Final time – initial time)}}$$

Thus rate is equal to the linear change in A610 divided by the duration in minutes. Prepare a summary table listing the respective rates for each treatment, including all controls.

3. Draw appropriate conclusions from the data in your summary table, especially with regard to the effects, if any, of each of the herbicides on the Hill Reaction compared to the controls.

4. Special conclusions should be made regarding the specific sites of action within the electron transport chain for each of the herbicides. For example, does paraquat accepts electron before DPIP site (DPIP accepts near PSII)?

5. The known mutants of photosynthetic electron transport chains and/or herbicide resistant mutant plants can be used to elucidate this pathway. Elucidate your expectations from using these mutants for determining the photosynthesis rate.