Plant Physiology Lab Manual
Fall Semester 2012

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Biology 347L
Plant Physiology Lab

Learning Priorities for This Year's Lab

Priority One: Mastering the concept of how to "scientifically" investigate plant phenomenon

-what is your question?
-composing a testable hypothesis based on your question.
-designing an experiment that will test your hypothesis

Priority Two: How to make sense of collected data

-how to statistically analyze your collected data for drawing legitimate conclusions.
-how to portray your data
-supporting or refuting your hypothesis

Priority Three: Mastering experimental tools

-becoming technically proficient in using instruments and making measurements
-striving for quantitative measurements versus qualitative measurements

Priority Four: How to communicate your findings to others using a scientific format (research paper, poster, oral presentation)

-the presentation of your research project at the end

2012 - HOW TO Plant Physiology Lab Experiments Report Format

1. Investigative question.

This is a question we are asking about the plant process or phenomenon that leads us to set up our investigation?

Example: the sun vs shade grown leaf study.....our investigative question could be

"Are the leaves found in the shaded, low-light regions of a tree different with respect to their photosynthetic structure compared to light exposed leaves?

2. Hypothesis

Formulate a hypothesis about our question that is falsifiable/testable by experimentation (H_1) **–** This begins with a theory you may have on what is happening BASED on previous knowledge (this previous knowledge for our purposes can be meager but it must have some basis being scientifically truthful).

Example: Shade leaves adapt to low light by boosting chlorophyll levels and increasing QY (photosynthesis efficiency) in order to trap and make the most use of what little light they do receive when compared to light exposed leaves.

3. Experimental design.

Testing the hypothesis experimentally: the experimental design. *For purposes of this class, I will not require technical details. You need to include only those aspects of how your data was collected.*

This can be done by a couple of paragraphs or simply using "bullets" to list the sequence of steps you did to get your data:
-harvest 4 shaded leaves and 4 sun exposed leaves into container of water
-place in dark cabinet for 30 min
-remove from water, daub with paper towel
-use hand held digital chlorophyll content meter
-clamp on center underside of leaf and make measurement

4. Results.

This will consist of graphs or tables NOT BOTH. In most cases, when you are able to graph your data using a bar graph, it will not be necessary to include a table version of your data.

-use Excel, online calculators, hand held calculators to convert your data into

✓ mean
✓ standard error or standard deviation
✓ students t-tests;

-then plot data graphically using a bar graphs with error bars, labeled x and y axis, a brief figure legend.
(see example figure as a "template" for how to compose your plots

5. Conclusion

Begin this section by revisiting what you stated in your hypothesis and then reflect on in writing. Consider whether or not your findings (the data) support or refute the hypothesis? Are differences between the treated and control statistically valid? Use the p value to validate what you can or cannot conclude from your experiment. If your hypothesis was falsified by your experiment, provide an alternative theory that accounts for your findings.

Plant Physiology Fall 2011
Lab 2

Today's activities:

1. Intro to lab:

-lab manual
-benches, working in pairs
-safety issues
-lab schedule
-overall learning goals
-our D2L site
-lab assessments and grades

2. First lab: measuring chlorophyll in leaves

Plant Physiology Fall 2012
Lab 1

Purpose:

-to learn the concept of how to conduct an experiment in plant physiology

Today, we will break the ice by doing a whole-class study of the photosynthetic properties of tree leaves. We will all go through the motions of how to set up an experiment by first formulating a question and then composing a testable hypothesis based on this question.

After this, we will design an experiment that will test our hypothesis, that is, an experiment that will yield data for supporting or refuting our hypothesis. These and other steps for our first "study" are listed as follows.

1. formulate a question about what you wish to investigate
2. compose testable hypothesis based on question
3. design the experiment
4. obtain data
5. crunch data (analyze data)
6. make conclusion based on data if hypothesis supported or refuted

What we are going to investigate today: How tree leaves from shaded and sun exposed parts of the tree differ with respect to chlorophyll content and composition.

Instrument you will use today:

✓hand-held chlorophyll content meter - measures the relative chlorophyll content in leaves

a. hand-held chlorophyll content meter Chlorophyll is the pigment in chloroplasts that absorbs light energy for use in the photosynthetic process. The amount of chlorophyll in leaves may be an important indicator of how much light the leaf can trap or absorb and use for photosynthesis.

WORK IN PAIRS----LAB WORK TO DO:

Each pair of bench partners will have ready:

-plastic container filled with about 2 inches of room temperature tap water
-scissors
-a sharpie marking pen
-3 paper towel squares
-eight 15 mL graduated conical plastic tubes
-leaf punch

Part I. Leaf Safari.

With me leading, we will all go outside to the parking lot across the street and I will give a quick demo on how to pick your leaves. Then I will assign you a tree to sample your leaves from.

-take scissors and harvest 4 shade and 4 sun leaves.....use a sharpie to mark an **"L"** on a sun leaf and a **"D"** on a shade leaf

-place into container and return to lab

Part II. Measurements

Take turns with the hand-held <u>chlorophyll meter</u>

-just before you are ready to measure, remove leaves from water, daub off moisture with paper towel,

-make on measurement on leaf----clamp on so the measure is from the bottom of the leaf

Record your data in the data table attached to this lab.

Place the leaves back into the container and proceed to **part III**, the chlorophyll extraction procedure.

Part III. Chlorophyll extraction.

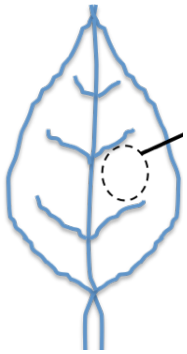
You will prepare a chlorophyll "extract" for each leaf and save it for next week. This is done by using a leaf punch to excise a circular portion from the leaf that you did the clamp-on measurements and placing it into a tube of ethanol. The ethanol leaches the chlorophyll out of the leaf. Next week, we will do a spectrophotometer based quantitative assay of the chlorophyll.

A. Directions.

1. get 4 x15 mL graduated conical tubes (one for each of your leaves that you harvested and measured, e.g., if you did "sun", get 4 x Tubes.

2. using an Ethanol squirt bottle, fill each tube to the 7 mL mark ----use the meniscus method for precise volumes.

3. Punch out a circle of a respective sun or shade leaf corresponding to the part of the leaf where you did the clamp-on measurement:



4. using forceps, stuff the leaf piece into the tube so it is immersed in the ethanol. Screw the cap on and shake for 3-4 secs. Place in tube rack. When done with all of your leaves, place in tube rack into the fridge for next week.

BE SURE YOUR TUBES ARE LABELED ACCORDINGLY!

Data Sheet for Leaf Measurements

Names:_____

_____

Name of tree you obtained leaves from:_____

I. Chlorophyll Content Meter Data

Record your numbers in the spaces below.

	Shade	*Sun*
leaf 1		
leaf 2		
leaf 3		
leaf 4		

Lab Report for Chlorophyll Content of Shade versus Sun Leaves

Name: _____

1. Investigative question.

2. Hypothesis

3. Experimental design

4. Results

We will do this section next week as a class after introducing how to statistically process raw data for displaying as a graphical figure. Ultimately, we will calculate the following values:

✓ mean
✓ standard error or standard deviation
✓ students t-tests;

5. Conclusion

We will do this section next week as a class after we process your data in #4 above.

Plant Physiology Fall 2012
Lab 1

End of lab directions

What you need to turn in today:

- your data sheets with your chlorophyll meter numbers
(sign both names and turn in one sheet)

What you need to turn in next week: **Nothing**

Plant Physiology Fall 2011
Lab 2

Today's activities:

1. Learning how to use adjustable pipettors..

2. Assessing pipetting skill by constructing a protein standard curve via the protein-binding dye assay method.

3. graphing a protein standard curve using excel.

Plant Physiology Fall 2012
Lab 2

Measuring the amount of protein in solutions using a protein binding dye assay

This exercise follows our lesson on using adjustable micropipettors. It will reveal whether or not you have mastered the pipetting skills necessary for the labs ahead. Should you fail in your first attempt, I will have you try again until acceptable results are achieved.

Discussion before starting

a. what is protein
b. discussion of the importance and use of measuring protein in plants
c. examples of proteins in plant cells and tissues
d. what is a standard curve? what is a protein standard curve? what is a protein standard?

Establishing a standard curve is necessary for determining the concentration of protein in an unknown sample. The "standard" in the term "standard curve" refers to two concepts:

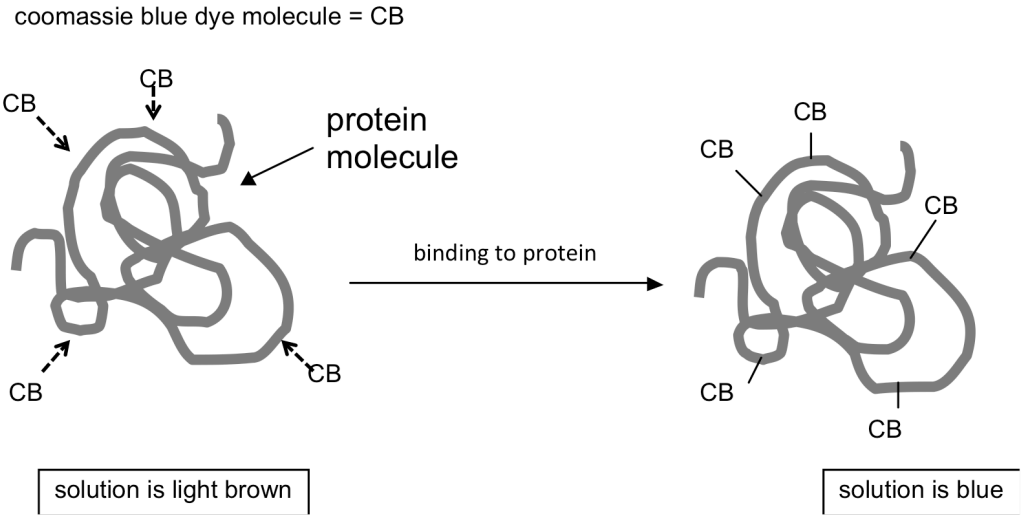
(i) it is a standardized, reusable, semi-permanent reference to compare solutions of unknown protein amount against

(ii) a **generic** kind of protein is used as the protein source for making the standard curve

For example, bovine serum albumin (BSA), is blood protein found in cows blood, has been adopted by scientists as a "generic" protein molecule for making a protein standard curves in scientific research.

e. the protein dye-binding assay

The Bradford method for determining the concentration of protein in a biological material is the most widely used procedure in all of biology. It is referred to as a “dye binding” assay because the active ingredient in the Bradford reagent is a dye called coomassie blue, or CB for short. When CB binds to a protein molecule, it changes color from brown to blue:



This color change is measured using a spectrophotometer set at a wavelength of 595 nanometers. The principle by which we can use the spectral (light absorption) characteristics to "quantitate" the concentration of a molecule is called **Beer's law**. This means we assume that as we add more protein to our solution, it will turn

blue in a linear or **proportional** manner. So if I add 1 μgram of protein to a 1 ml solution of Bradford, it will give and absorbance of 0.1. If I add 2 μg to the Bradford solution, it will give an absorbance of 0.2 and so on.

Part I: "constructing" a protein standard curve

Each pair of bench partners will have:

✓a container of 1.5 mL microcentrifuge tubes
✓microcentrifuge tube racks
✓a 50 mL tube of Bradford protein assay reagent
✓sharpie marking pens
✓a 96-well plastic micro titer dish

Each person does a curve.

-place 5 X 1.5 ml microfuge tubes in a microfuge tube rack and label
label duplicate tubes with sharpie pen with the numerals 0, 2.5, 5.0, 7.5, 10.0

1. to each tube in the tube in rack, add 1000 μL Bradford solution to each tube (using P-1000 pipetman)
.....change tip only if the solution appears to be sucking up into the shaft of the pipetman

2. Add the following amount of BSA protein standard solution using a P-20 pipetman as follows:

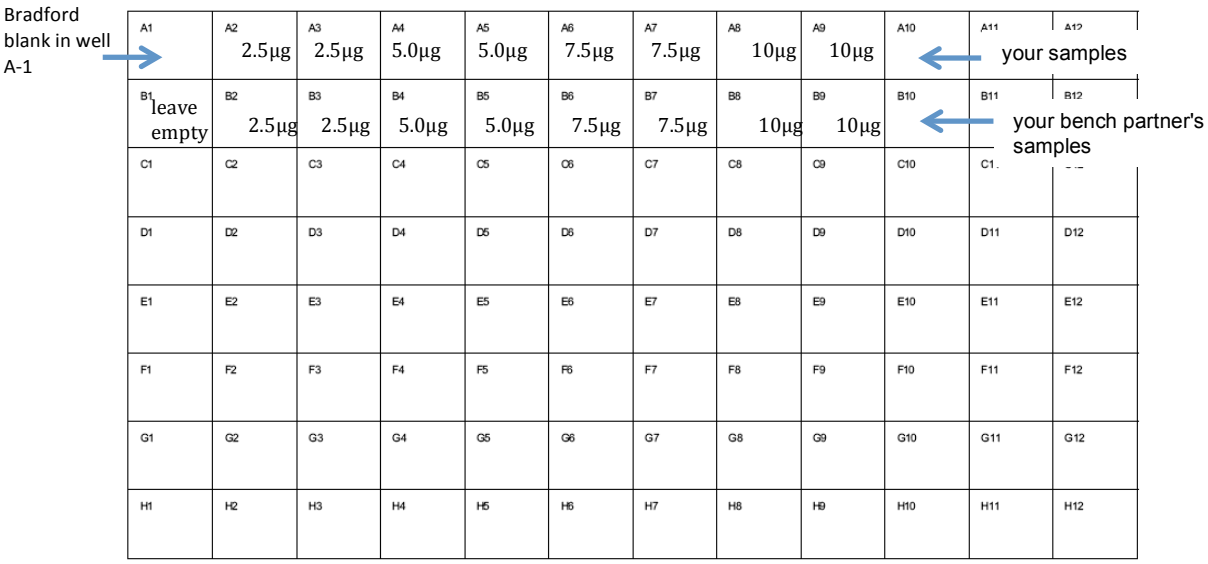
(change tip in between)

tube number (in duplicate)	μl of BSA std to add
0	0 μL
2.5	2.5 μL (= 2.5 μg protein)
5	5 μL (= 5.0 μg protein)
7.5	7.5 μL (= 7.5 μg protein)
10	10 μL (= 10 μg protein)

3. snap cap close and mix tubes by briefly vortexing

4. let sit 5 min *(OK to let sit for up to an hour)*

5. Using a P-1000, pipet 300 µl of from each assay tube into the pre-arranged well of a microtiter plate for subsequent assay as follows:



6. Proceed to assay your samples on the Bio-tek Plate reader.....I'll give a demo

7. Enter your numbers on enclosed the lab 3 data sheet

Part II. Plotting a protein standard curve using Excel

a. go to our D2L and see the step-by-step example on "how to plot a protein standard curve using Excel" (go to one of the computer labs around Science Lab or Hagen that has a printer during lab).

1. plot and print the curve as specified on the D2L example.

2. Staple this to your data sheet and sign your name. You can turn it in at the end of lab *IF* I approve that your pipetting is acceptable. Let's say to pass this test your standard curve must have an R^2 value ≥ 0.90.

DONE!

LAB 3 Data for Bradford protein assay **Name:** _____

a. Record your absorbance values in the following table

Protein Standard Sample	A_{595} (record duplicate values)	Average A_{595}
2.5 μg BSA		
5.0 μg BSA		
7.5 μg BSA		
10.0 μg BSA		

b. Attach your protein standard curve and list the following values obtained from the Excel plot:

✓std curve slope formula (e.g. y = 0.0978x):_____

✓std curve R^2 value (e.g., 0.923): _____

c. Use your std curve slope formula to compute the protein amount for the following "hypothetical" absorbances:

A_{595} = 0.023 = _____μg protein?

A_{595} = 0.145 = _____μg protein?

A_{595} = 0.322 = _____μg protein?

Example: for an A_{595} = 0.220. Use the formula computed from your standard curve.

y = 0.0978x

where y = A_{595} and x = μg protein

0.220 = 0.0978 (x)

x = 2.25 μg protein

Plant Physiology Fall 2012
Lab 2

End of lab directions

What you need to turn in today: your protein assay data sheet with the plot of your protein standard curve attached.

What you need to turn in next week (Sept. 20): A lab report for **Lab 1** (leaf chlorophyll content) using two sets of data: your data and the collective class data for the tree species you measured. Use the standard *Lab Report* form. Download the form from the D2L site. Complete all sections as described in the HOW TO lab report guide in the appendix section of this manual. You may include any content from the sections we initiated today.

✓Use the word-doc form to enter your sections 1-5 so that the section headings remain. Insert the finished figure into the word-doc (no larger than 1/2 page).
✓Try to limit your report to 2-3 pages (not mandatory).
✓Typed not hand written

YOU WILL DO TWO GRAPHS that will be composed into SEPARATE FIGURES

-one figure will be yours and your partners data (4 measurements per shade, light)
-one figure will be the collective class data for your tree species

In particular, I will be focusing on how well you follow the specified format for composing the figure from your data. It is important that you follow the RULES we are using for all our figures (see figure check-list in appendix materials).

In sum, you must:

✓Use a bar-graph plot
✓This will have two data bars per each figure, sun vs. shade
✓Use as a label for the Y axis: chlorophyll content per cm^2 leaf area

Plant Physiology Fall 2012
Lab 3

Today's activities:

1. Introduce principles of UV-VIS absorption spectroscopy &-spectral analysis of chlorophyll

2. Light-absorption spectroscopy of chlorophyll extracts prepared in lab 1 for measuring:

- leaf chlorophyll concentration in shaded versus sun exposed leaves (from lab 1)

- the relative amount of chlorophyll type-a and chlorophyll type-b shaded versus sun exposed leaves (from lab 1)

Plant Physiology Fall 2011
Lab 3

Analysis of chlorophyll in tree leaves - part II

What we are going to investigate: How tree leaves from shaded and sun exposed parts of the tree differ with respect to chlorophyll-type (type a versus type b) and the amount of total chlorophyll per unit of leaf.

Instrument you will use today: UV-Vis spectrophotometer - Biotek™ plate reader.

Directions

WORK IN PAIRS

Each pair of bench partners will have ready:

- your leaf samples in tubes of ethanol from lab one
- p 1000 micro pipettor
- a sharpie marking pen
- a 96-well plastic micro titer dish
- a squirt bottle of 100% EtOH

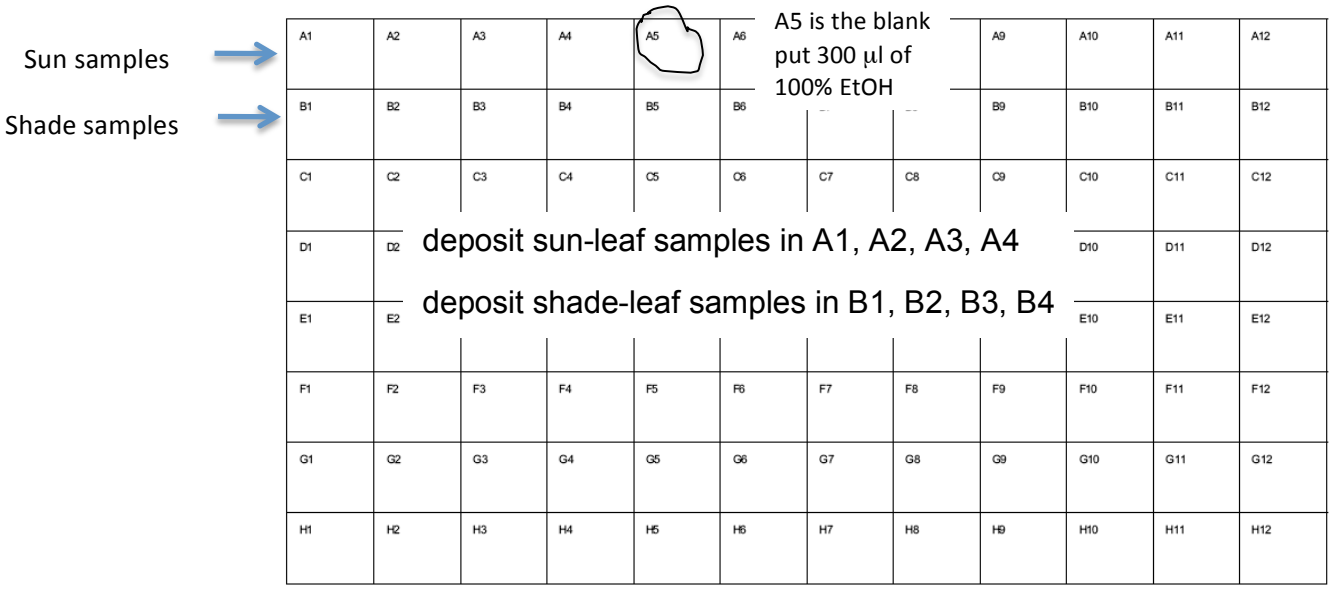
The idea is to transfer an aliquot of your chlorophyll solution from the leaf-tubes into a specified well of the 96-well micro titer dish. You will then insert the plate into the pre-programmed Biotek plate reader-spectrophotometer, press a button on the instrument, and obtain absorbance readings from your chlorophyll samples. Get a print-out of your absorbances back to your bench for calculations.

Here are step-wise instructions:

1. get your leaf-tubes in ethanol from week one (stored in freezer).

-the chlorophyll should have leached out of the leaves leaving a pale white leaf piece and a fairly green clear solution.

2. Using a p1000 micro pipettor transfer 300 μL of the green chlorophyll solution into a pre-designated well according to the diagram below.....use a new pipet tip for each sample transfer



3. Take plate over to the plate reader for an absorbance reading at a wavelength setting of 649 nm and another reading at 665. This will already be pre-programmed in the plate reader so you will simply need to push the "read plate" button. I will give a demo on how to do this. Once you have read your plate and obtained the Absorbance values for each sample as a print-out, take back to your bench.

-Proceed to calculate chlorophyll type-a to chlorophyll type-b ratio as described in step 4 and total chlorophyll per unit of leaf (step 5).

4. For each sample, calculate the chlorophyll a to chlorophyll b ratio and enter these on the pre-printed data entry sheet.

For each sample, you have obtained two absorbance readings: one at λ649 nm and another at λ665 nm. This is abbreviated as A_{649} and A_{665}. ***For example,*** if from one of your chlorophyll solutions you measure an absorbance of 0.122 at the wavelength setting of 649 nm. Thus, your A_{649} = 0.122 for this particular chlorophyll sample.

To find out the concentration of chlorophyll types in the chlorophyll solution, we will use what is termed an "empirical formula". An empirical formula is one in which the coefficients used in the formula are obtained by physical experimentation in the lab rather than by an abstract theory. In our case for chlorophyll an empirical formula was constructed by two researchers by the name of *Wintermans and Demott. They derived what is called an empirically determined formula for calculating the combined amount of chl a and chl b in ethanol. They did this by mixing known amounts of pure chl a and pure chl b together in ethanol and then measuring the absorbance values of the resulting mixtures. So, Wintermans and Demott have done the hard work for us. We need only to measure absorption of our chl solution at two specific wavelengths, plug these into the formula, and *crank* with our calculators.

chlorophyll type concentration formulas:

chl a = (13.7 x A_{665}) - (5.76 x A_{649}) = μgrams of chl a per mL of solution

chl b = (25.8 x A_{649}) - (5.76 x A_{665}) = μgrams of chl b per mL of solution

[1]Wintermans J.F.G.M., De Mots A. (1965) *Biochem. Biophys. Acta* **109**: 448-453

Example - assume you of obtained the following readings for a chl sample

A_{649} = 0.123

A_{665} = 0.224

How much chl a is in the sample? *Solve for chl a equation above:*

chl a = (13.7 x A_{665}) - (5.76 x A_{649}) = μgrams per mL of solution

↓

chl a = (13.7 x 0.224) - (5.76 x 0.123) = μgrams per mL of solution

↓

chl a = (3.07) - (0.71) = μgrams per mL of solution

↓

✓**chl a = 2.36** μgrams per mL of solution

Now, do the similar calculation for chl b using the formula above:

chl b = (25.8 x A_{649}) - (5.76 x A_{665}) = μgrams of chl b per mL of solution

chl b = (25.8 x 0.123) - (5.76 x 0.224) = μgrams of chl b per mL of solution

chl b = (3.17) - (1.29) = μgrams of chl b per mL of solution

✓**chl b** = **1.88** μgrams per mL of solution

Now, simply make a ratio with these two numbers and divide to get a single number, the chlorophyll a/b ratio.

$\frac{2.36}{1.88}$ = **1.25**

this is the value you would enter in the data sheet

5. Using the following formula and the values from # 4 above, compute the total chlorophyll per unit of leaf for each sample and enter these on the pre-printed data entry sheet.

a. compute total chl conc.

chl a (μgrams/mL) + chl b (μgrams/mL) = total chl (μgrams/mL)

b. compute the total μgrams of chl that was leeched from the leaf circle into the tube's 7 mL of EtOH

μgrams total chl[1] x 1/300 μL x 7 mL x 1000 μL/mL

[1]from #1 above

c. compute the area of leaf circle

area of a circle is πr^2

Since we used a 1 cm diameter leaf punch, r, the radius is 0.5 cm.

Thus, the area of the circle is $(3.14)(0.5)^2 = 0.79\ cm^2$

d. Finally, compute the total chlorophyll per unit of leaf

total μgrams of chl (from #2)/leaf area (cm^2)

= total μgrams of chl/0.79 cm^2

= **μgrams of chl/ cm^2** *this is what you enter on your data sheet*

Example: From the hypothetical example in 4 above, chlorophyll a and b concentration was:

chl a = 2.36 μgrams per mL of solution

chl b = **1.88** μgrams per mL of solution

a. total μg chlorophyll per mL = 2.36 + 1.88 = **4.24**

b. compute the total μgrams of chl =

4.24 x 1/300 μL x 7 mL x 1000 μL/mL = **98.9** μgrams of chl

c. leaf area = 0.79 cm^2

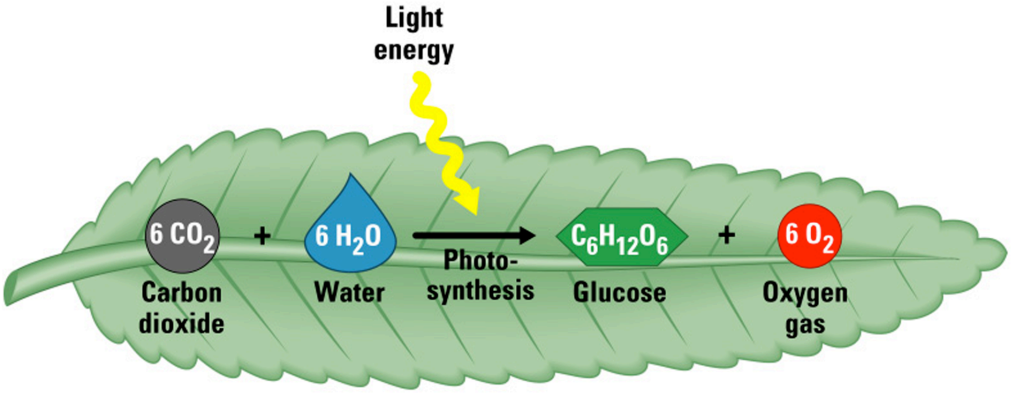
d. compute the total chlorophyll per unit of leaf

98.9 μgrams of chl/0.79 cm^2 = **125.2 μgrams of chl/ cm^2**

this is the value you would enter in the data sheet

Lab 2 reference: why leaves are green, the absorbtion spectra of chlorophyll a and b

-photosynthesis is energized by the radiant energy contained in red and blue light.



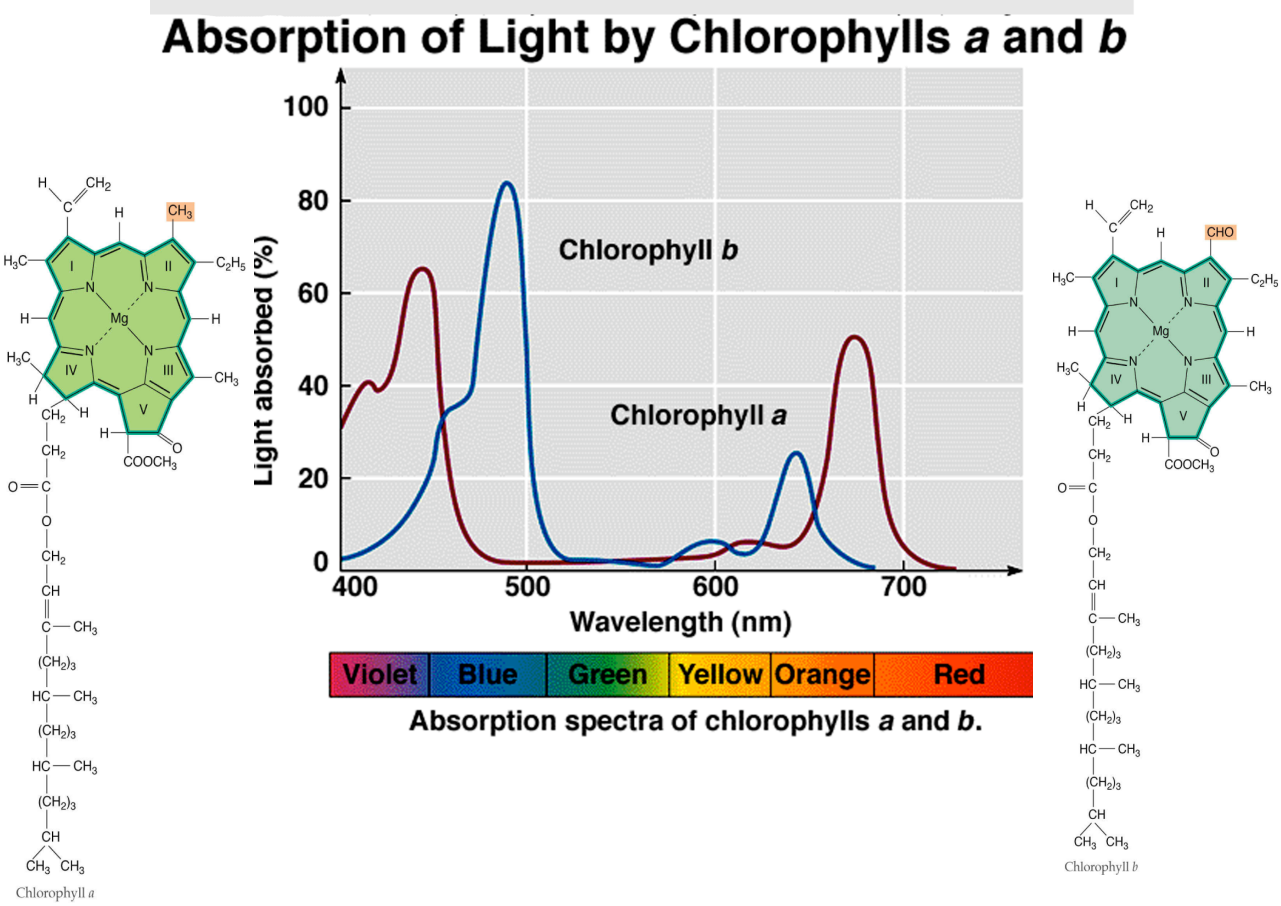


Table 12-4 Some Photosynthetic Characteristics of Three Major Plant Groups.

Characteristics	C-3	C-4	CAM
Leaf anatomy	No distinct bundle sheath of photosynthetic cells	Well-organized bundle sheath, rich in organelles	Usually no palisade cells, large vacuoles in mesophyll cells
Carboxylating enzyme	Rubisco	PEP carboxylase, then rubisco	Darkness: PEP carboxylase light: mainly rubisco
Theoretical energy requirement (CO_2:ATP; NADPH)	1:3:2	1:5:2	1:6.5:2
Transpiration ratio (H_2O/dry weight increase)	450–950	250–350	18–125
Leaf chlorophyll *a* to *b* ratio	2.8 ± 0.4	3.9 ± 0.6	2.5–3.0
Requirement for Na^+ as a micronutrient	No	Yes	Yes
CO_2 compensation point (μmol mol^{-1} CO_2)	30–70	0–10	0–5 in dark
Photosynthesis inhibited by 21% O_2?	Yes	No	Yes
Photorespiration detectable?	Yes	Only in bundle sheath	Detectable in late afternoon
Optimum temperature for photosynthesis	15–25°C	30–47°C	≃35°C
Dry matter production (tons/hectare/year)	22 ± 0.3	39 ± 17	Low and highly variable
Maximum on record[a]	34–39	50–54	

[a]Monteith, 1978.

Source: Modified from table in Black, 1973.

The more rapid photosynthesis of C-4 species under high irradiances results in a lower water requirement per gram of dry matter produced (a higher **water use efficiency**), but CAM plants have much lower requirements than either C-4 or C-3 species. Often C-4 species can also get by with less nitrogen than C-3 species (Brown, 1978). Table 12-4 compares some of these and other photosynthetic characteristics of C-3, C-4, and CAM plants, most of which are described in Chapter 11 or in the following sections. A good review of the ecophysiology of C-3 and C-4 plants is an article by Pearcy and Ehleringer (1984).

Adaptations to Sun and Shade

In trees, shrubs, and to some extent in herbaceous plants, many leaves develop in the shade of others and attain during development characteristics that are much like those of true shade plants (Corré, 1983; McClendon and McMillen, 1982). These are called **shade leaves**, as opposed to **sun leaves** that develop in bright light. In dicots, shade leaves are typically larger in area but thinner than sun leaves. Sun leaves become thicker than shade leaves because they form longer palisade cells or an additional layer of such cells (Fig. 12-5). On a weight basis, shade leaves also generally have more chlorophyll, especially chlorophyll *b*, mainly because each chloroplast has more grana than do those of sun leaves. In addition, grana of *Alocasia* and certain other shade plants develop far more thylakoids in the grana, up to 100 per granum (Björkman, 1981). On the other hand, chloroplasts of shade leaves have less total stroma protein, including rubisco, and probably less thylakoid electron transport protein than do sun leaves (Boardman, 1977; Björkman, 1981). Thus shade leaves invest more energy in producing light-harvesting pigments that allow use of essentially all the limited amount of light striking them. Furthermore, chloroplasts in leaves exposed to deep shade become arranged by phototaxis within the cells in patterns that maximize light absorption (Section 20.10). The petioles of dicots also respond to the direction and intensity of light by bending (Section 19.2), causing the leaf blades to move into less shaded regions. All these factors allow net CO_2 fixation under low irradiance levels with minimum energy cost to produce and maintain the photosynthetic apparatus.

Data entry sheet for leaf chlorophyll-part II

Plant Physiology Lab 3-2012 **Names:** _____

_____

What is the name of the tree that you used for your measurements? _____

Enter your computed chlorophyll a to b ratios in the tables below.

YOUR DATA	
leaf chlorophyll-type expressed as a ratio of chlorophyll a to chlorophyll b (chl a/chl b)	
Shade leaf samples	*Sun leaf samples*

Enter your computed chlorophyll concentration data in the tables below:

Your DATA	
leaf chlorophyll concentration expressed as μgrams total chl per cm^2 leaf area ($\mu gram\ chl\ /cm^2$)	
Shade leaf samples	*Sun leaf samples*

Plant Physiology Fall 2012
Lab 3

End of lab directions

What you need to turn in today:

-A lab report for **Lab 1** (leaf chlorophyll content).
-Completed **data sheets for today's chlorophyll measurements**

What you need to turn in next week (Sept. 27): **Nothing.**

-The lab report for today's lab will be due in two-weeks time on **Oct. 4.** I will tabulate the data as a class. We will use the collective class data for you to do your lab report for lab 3.

Plant Physiology Fall 2012
Lab 4

Today's activities:

1. New experiment: quantitative measurement of soluble protein in salad greens.

"Which salad leaf-green is highest in protein? - protein assay of assorted salad greens from the grocery store."

2. using mortar and pestles for homogenizing plant tissue

3. using the "fresh weight" unit measure as normalizer

4. preparing a soluble protein extract from leaf tissue

5. computing soluble protein content of salad greens using a protein standard curve

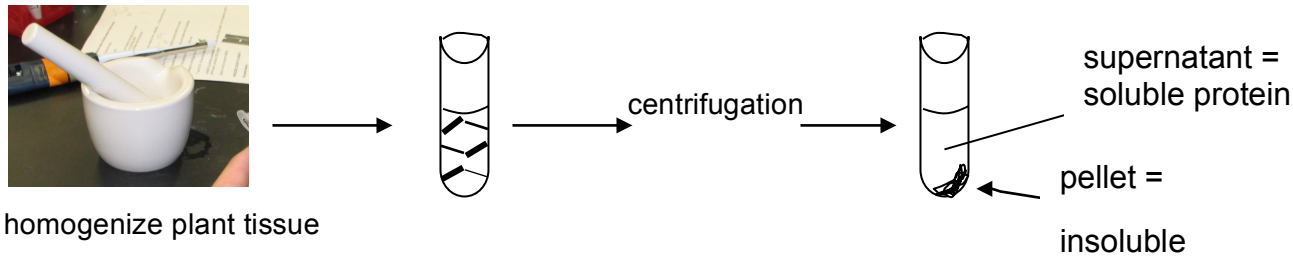
Plant Physiology Fall 2012
Lab 4

Which salad leaf is highest in protein?-using the Bradford protein assay to measure protein amount in plant tissue.

Discussion before starting.

a. measuring plant processes using the normalizing unit of plant tissue "fresh weight".
b. how much of the plant is water?
c. other normalizing units used in quantitating plant processes - comparing apples to apples
d. nutritional importance of leafy greens
e. how many replicate measurements? duplicates, triplicates, quadruplicates???
f. soluble versus insoluble protein in plant tissues
g. compose a hypothesis for your lab report due next week. - let's think about this together.

Today, we will not be quantitating a plant *process*, but a type of quantitative measure that plant physiologists routinely use, namely determining the soluble protein content in plant tissues. The term soluble protein relates to the fact that protein in cells exists in two fractions, soluble and insoluble. A cell protein is soluble if it originates from an aqueous portion of the cell such as the cytosol. It is insoluble if the protein is embedded in cell membrane, such as a protein found in the plasma membrane. When one homogenates the cells using a mortar and pestle, the soluble and insoluble proteins can be separated using centrifugation:



Our goal today is to measure the **soluble protein concentration** in plant tissue using the Bradford protein assay.

This is accomplished by first determining the protein concentration in a sampling of plant tissue and then back calculating to the amount of protein contained in ***fresh-weight** of the plant tissue from that was homogenized in the mortar and pestle:

*As plants are 95% or more H_2O, the measurement termed "fresh"-weight is a commonly used to as a biologically relevant reference measure of quantitating the amount of plant per process. Importantly, by fresh, we mean *"fresh from the plant"* or, in other words, in its original condition and ***not wilted*** or ***dried out***.

I. Prepare soluble leaf tissue "extracts"

Work in pairs - each person will be assigned a leaf-green such as spinach or endive. Each person will prepare three separate leaf extracts for the type of leaf your are assigned.

Have ready at your bench:

✓ice bucket with ice
✓scissors
✓mortar and pestle
✓container of dispo transfer pipets

✓small beaker of water
✓a container of 1.5 mL microcentrifuge tubes
✓microcentrifuge tube racks
✓a 50 mL tube of Bradford protein assay reagent
✓sharpie marking pens
✓a 96-well plastic micro titer dish

Procedure: - keep plant material in a large plastic weigh boat positioned on top of the ice in your ice bucket to keep leaves from drying out.

1. cut your leaves into small pieces (~1/2 inch x 1/2 inch [1 cm x 1 cm]) and store on ice. Randomize the leaf pieces by mixing the pieces before weighing.

2. use the pan balances on the side bench - weigh around 0.5 grams into a small plastic weigh boat record the exact "fresh weight" in the space designated on the data sheet.

3. place the weighed tissue pieces into a mortar AND: homogenize the plant in mortar and pestle:

-add 3 ml water using a p 1000 pipetman - add 3 X 1 ml

-use pestle to homogenize into to a puree -takes about 30-60 seconds

- suck out puree with a wide-tip dispo pipette and fill up a pre-labeled 1.5 ml microfuge tube. (also label this tube with your initials in case you are sharing the centrifuge with others.)

4. When you have homogenized all three of your triplicate samples spin the tubes for 5 min in the microfuge on highest speed setting...be sure and balance tubes accordingly

5. Using a wide-tip dispo pipette transfer some of the supernatant to a new labeled 1.5 ml tube and place in tube rack..... be sure to avoid transferring any of the pelleted gunk;

II. Bradford Protein Assay of Leaf Extracts

STEP 1. For each plant leaf extract you have prepared, label two 1.5 ml microfuge tubes to serve as duplicate protein assays; For example, you prepared three tubes of extract for the same kind of leaf, you will need a total of six 1.5 mL tubes;

STEP 2. Add 1.0 ml Bradford reagent to each tube using the P-1000 pipetman
ALSO,...be sure to make-up a single separate blank or background tube with 1 ml Bradford solution. This tube will have NO protein sample added to it.

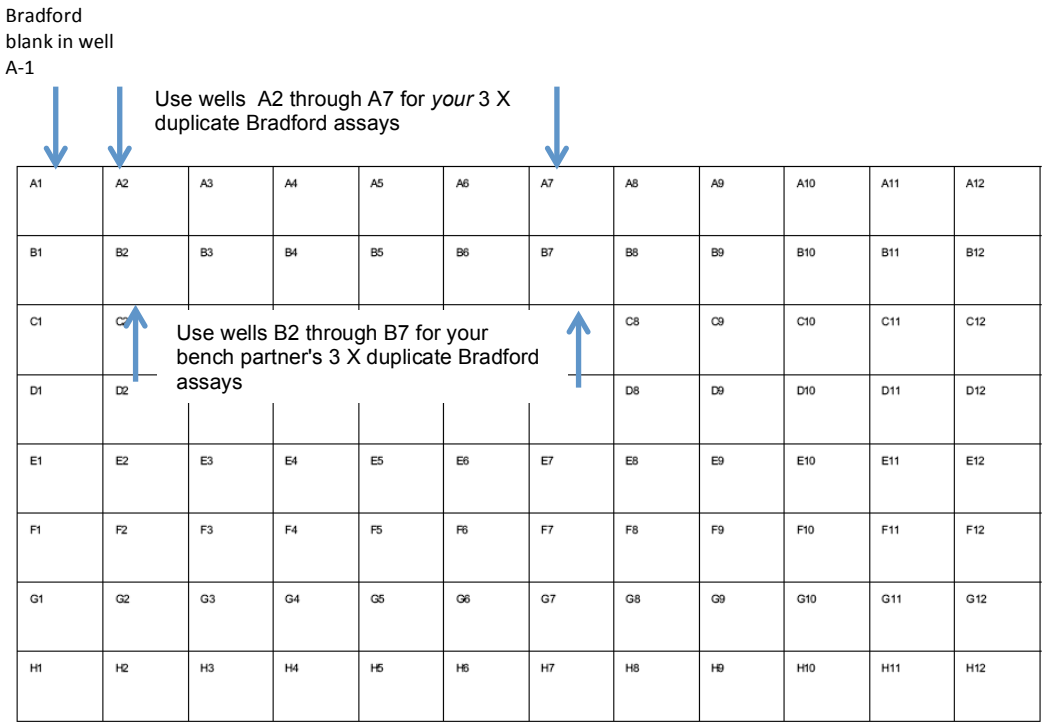
STEP 3. Using a P-20 pipetman, add 10 μl of plant leaf extract to the respective duplicate 1 mL Bradford assays

STEP 4. snap tube closed and vortex a few seconds to mix

STEP 5. let sit at least 5 min.....the 5 min mark is a minimum time---the color change is stable after this

-READY TO "READ" SAMPLES……

STEP 6. Using a P-1000, pipet 300 µl of from each assay tube in a pre-arranged well of a microtiter plate for subsequent assay of your samples on the Bio-tek plate reader



STEP 7. Proceed to the plate reader and scan your samples with the plate reader, save results to an excel file and obtain a printout for your records.

III. Calculations - determine the protein content in plant leaves

STEP 1. Using your protein standard curve from the previous week to convert your raw absorbance readings into protein amount.

Consult your Bradford curve from last week. You calculated a std curve slope formula. Use this formula (or a similar one we will announce in lab for EVERYONE to use this day---TBA).

It resembles something like: "y = 0.0932x" .....

y is the value corresponding to an aborbance from your assayed sample
x is the value corresponding to the protein amount in µgrams in your sample
0.0932 is the std curve slope factor

Find the protein amount for each assayed sample by solving for x *your* std curve formula (not the example one above).

1. start by averaging the two absorbance values you recorded for each of duplicate assay samples using a calculator.- enter this value in the space designated on your enclosed data sheet

2. plug the averaged absorbance values into your std curve slope formula and calculate the protein content in your leaf-extract Bradford assay

Example: one of your spinach leaf extracts was Bradford assayed in duplicate. The averaged absorbance was 0.236. Use the formula to determine the corresponding protein amount in µgram of protein.

y = 0.0932x 0.236 = 0.0932x è 0.236/0.0932 = x è 2.53 µgram protein = x

- enter these values into your data sheet

3. When you have completed all calculations in **2.** above, proceed to the final set of computations (below) that will give us the amount of protein in the leaf before you ground it up.

STEP 2. protein per leaf calculations

Plant Physiology 2010: Sample Calculation of Soluble Plant Protein Concentration

Your experimental procedure looked something like this:

This is an example calculation.. your numbers will be different

✓Starting fresh weight of plant = 0.34 grams

✓Grind in 3 ml of water

✓Add 10 μl of protein extract to duplicate tubes of 1 ml Bradford

✓Read and record absorbance at 595 nm

✓Use protein std curve slope formula for conversion of absorbance values to μ grams of protein

NOW YOUR READY TO START HERE: Now, from our previous example ABOVE, we found that 2.53 μg protein was IN that 10 μl aliquot of extract you added to the 1 ml of Bradford reagent. *You now want to quantitatively express (or compute) the protein content in this leaf as:*

μ grams of protein per mg of fresh leaf weight

1. First, back-calculate how much liquid was in the entire plant extract after you added your leaf to the 3 mL of H_2O and ground it to a puree:

In this example, this total amount of liquid in the entire plant extract is 3.0 mL+ 0.0.34 mL = 3.34 mL

Where did the 0.34 mL come from?

It represents the 0.34 grams of leaf material you ground up. Since plant tissue is >95% H_2O, we assume that it represents almost 0.34 mL of H_2O because, by definition, 1 metric gram wt = 1 mL of H_2O.

2. Next, calculate how much protein was in the WHOLE entire plant extract you ended up with in the mortar after grinding:

Calculate this as follows:

dimensional conversion

$$\frac{2.53\ \mu\text{gram protein}}{10\ \mu\text{L extract}} \times 3.34\ \text{mL total liquid extract} \times \frac{1000\ \mu\text{l}}{1\ \text{ml}} = 845\ \mu\text{g protein}$$

this is the volume of extract that you added to the 1 mL Bradford assay

3. Next, convert the original leaf weight (0.34 gr) from grams to milligrams:

$$0.34\ \text{gr} \times \frac{1000\ \text{mg}}{1\ \text{gr}} = 340\ \text{mg}$$

4. Lastly, determine the protein content in the intact leaf by dividing total μgrams of protein (from step 2) by the total "fresh weight" of the leaf (from step 3) :

$$\frac{845\ \mu\text{g protein}}{340\ \text{mg plant}} = 2.49\ \mu\text{g protein } \textit{per}\ \text{mg leaf } \textit{tissue}$$

This is the value you record in your data sheet

Lab-4 2012 **Name:**_____
Leaf Protein Content

Data sheet for reporting your leaf protein values

A. Record your A_{595} protein absorbance values below:

A_{595} *Absorbance*

leaf - 1
duplicate average_____

leaf - 2
duplicate average_____

leaf - 3
duplicate average_____

B. Record your converted absorbance-to-protein values below:

μg protein per 10 μL of leaf extract

leaf - 1_____

leaf - 2_____

leaf - 3_____

C. Record your protein per leaf values below:

μg protein/mg leaf fresh wt

leaf - 1_____

leaf - 2_____

leaf - 3_____

D. Record the class protein per leaf values below:

Class Data Leaf Protein (*μg protein/mg leaf fresh wt*)		
Spinach	**Iceberg**	**Romaine**

Plant Physiology Fall 2012
Lab 4

End of lab directions

What you need to turn in today: **your completed data sheet**

What you need to turn in next week, Oct. 4 : The lab report for **lab 3 (chl a/b, chl conc)**

Will I do a lab report for this lab, lab 4? : **Yes**, it will be due in two-weeks time on **Oct. 11.** I will tabulate the data as a class. We will use the collective class data for you to do your lab report for lab 4.

Plant Physiology Fall 2012
Lab 5

Osmosis - T.B.A.

This lab will be available for insertion into your lab manual one week before.

Plant Physiology Fall 2012
Lab 6

Today's activities:

1. New experiment: osmometer based measurement of the solute potential of plant cells.

Plant Physiology Fall 2012
Lab 6

Measuring Ψs, the solute potential using the plant sap method

Discussion before starting.

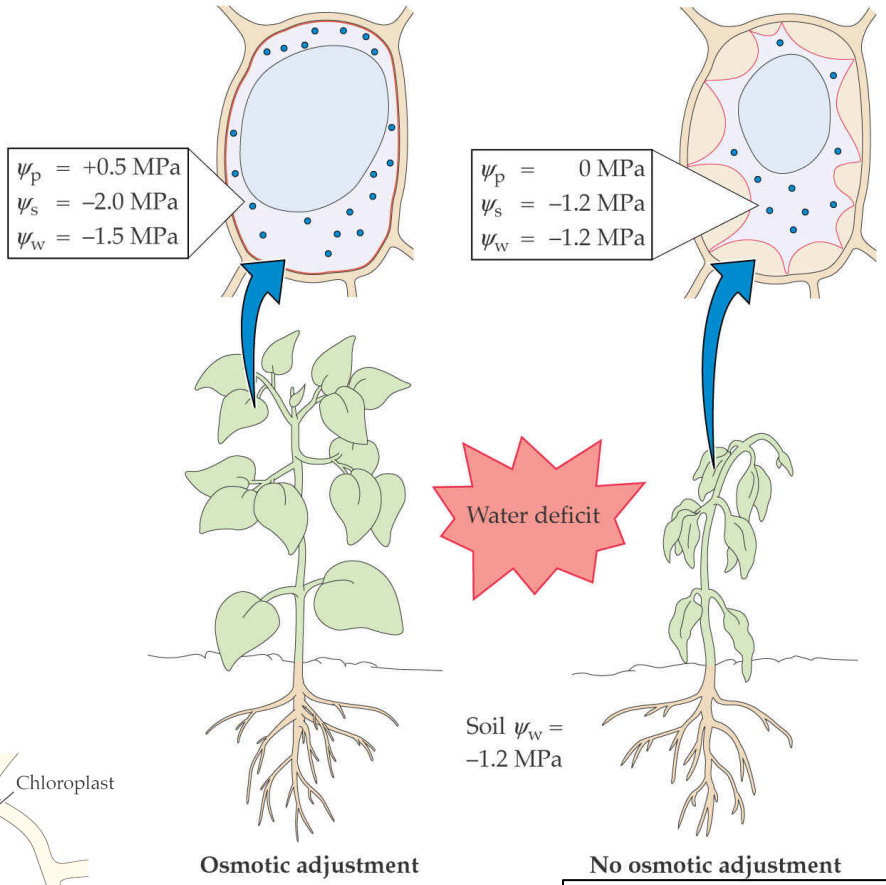
a. importance of knowing the water status of plants
b. what are the components of water potential?
c. what is the solute or osmotic potential, **Ψs**
d. what measurements of plant **Ψs** can reveal
e. plant responses to water deficits and osmotic adjustment mechanisms

Background:

Water potential **Ψw** is a measure or indicator of the water status of the plant. Plants must maintain a high degree of hydration for normal growth and development. Plant physiologists have measured **Ψw** for many species of plants under fully hydrated versus drought conditions. A key **Ψw** parameter uncovered in such measurements is termed the wilting point. This is when a plant that is experiencing a drought develops a water deficit so that it cannot replace the water lost by transpiration with water extracted by the roots from soil. The wilting point is when the water potential reaches a certain negative value that results in total loss of turgor so the plant wilts or droops.

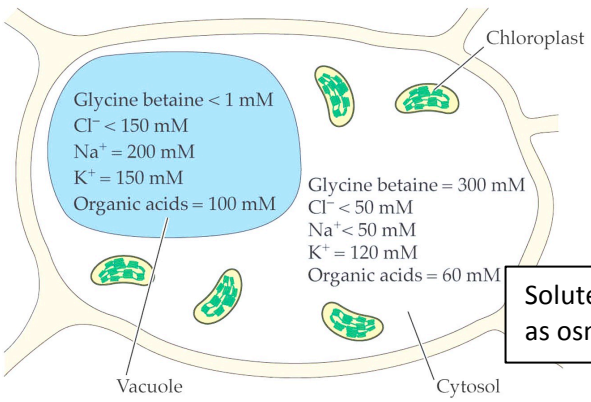
Plants can respond to a water deficit by adjusting their cell water potentials to better attract water into the cell. This has the effect or restoring turgor pressure, thus avoiding the wilting point.

The mechanism by which plants do this is termed osmotic adjustment which generates or produces an excess of soluble molecules in the cell (termed osmotic particles), since they have the effect of lower the **Ψs** of the cell or in other words, decreasing the concentration of water in the cell (see figure below).



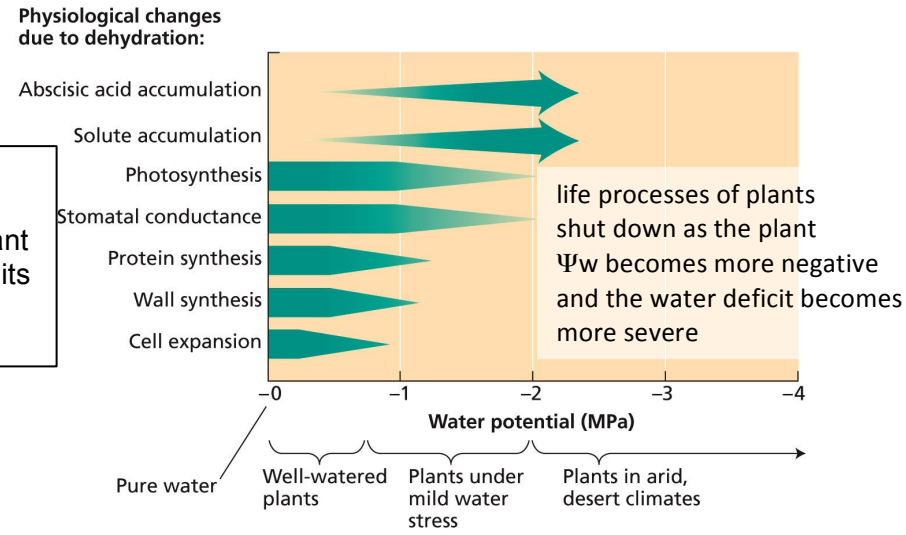
wilting point has been reached

Salt-stressed spinach leaf cell

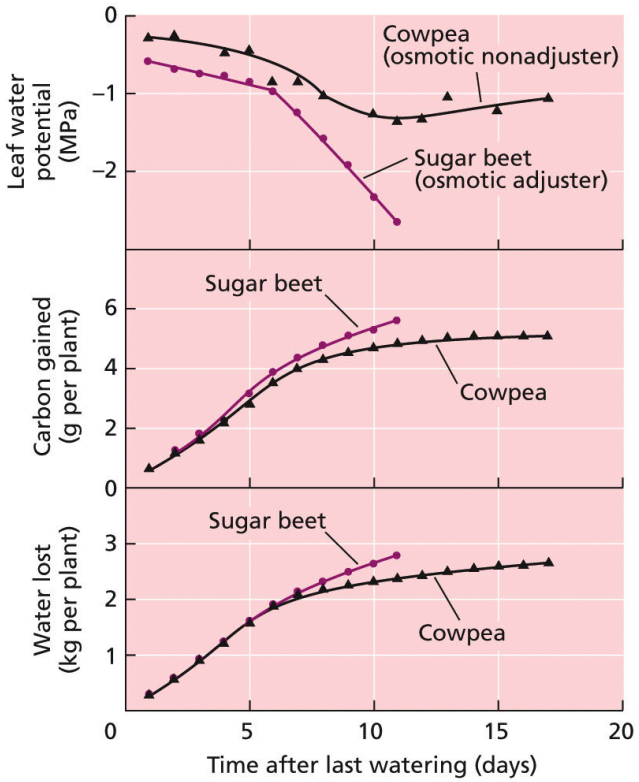


Solutes in plant cells that function as osmotic particles

Water deficit - when the water content of the plant is suboptimal for its life processes



Sugar beets are osmotic adjusters and show less ill effects when water deficits develop.



I. Measuring Ψs in leaves

Have ready at your bench:

✓mortar and pestle
✓small beaker of dH_2O
✓container of dispo transfer pipets
✓small beaker of water
✓a container of 1.5 mL microcentrifuge tubes
✓microcentrifuge tube racks
✓sharpie marking pens

Last week, we gathered leaves from outside, weighed them, and placed them in the freezer. Today, we wish to prepare what is called a cell-sap of the leaf for measuring **Ψs** in an instrument called a vapor pressure osmometer. The cell sap is simply the soluble contents or "juice" from the leaf minus insoluble material such as cell wall and membranes.

A. cell sap preparation

1. remove your frozen leaf from the foil and place entire into mortar.

-try cutting up into a few pieces

2. add 3 mL of dH2O and homogenize the leaf to a puree - some of us may wish to use a pinch of sand as a grinding aid.

3. using a transfer pipet, suck up some of the homogenate and approximately fill a 1.5 mL microfuge tube.

4. centrifuge the tube on high-speed for 3-5 min.

5. carefully remove the tube and transfer ~500 μL of the clarified "juice" into a new tube and place it into the tube rack on your bench.

READY TO MEASURE ON OSMOMETER.

B. Osmometer measurement

I will demonstrate how to do this simple measurement. Each measurement takes about 2 min. Please be patient and wait for your turn.

1. at the osmometer, place a white filter disc into the silver disc holder.

2. using the 10 μL pipet, transfer 10 μL of your plant juice onto the filter disc.

3. push the button and the instrument will withdraw the disc into the chamber.

4. wait ~2 min., the instrument will post your reading on the screen. This will be mmol of solute per Kg of H2O. This is also the milli molality of your plant juice.

-RECORD THIS IN THE DATA SHEET & Proceed to calculations.

Plant Physiology Fall 2012 **Name:** _____
Lab 6

Measuring Ψs, the solute potential using the plant sap method

Data Sheet

Name of plant where leaf came from.	
Fresh weight of leaf	
Osmometer reading (mmol/Kg H2O) or (mmolal)	
Ψs of your leaf (mPa)	

Show calculation for the Ψs of your leaf:

Plant Physiology Fall 2012
Lab 6

Calculation for determining the Ψs of your leaf:

Example.

Marilyn's poison ivy leaf weighed 0.23 grams. Her osmometer reading was 122 mmol/Kg H2O.

What is the Ψs of the leaf as it was when it was attached to the plant?

(i) First, convert the osmometer reading into molality. This is easy because the reading mmol/Kg H2O is also millimolal ($1x10^{-3}$ molal).

(ii) Then, use the Van't Hoff equation for ideal gases to convert the molality into osmotic potential:

$\Psi s = -C\gamma RT$

C = concentration of solute in molality
γ = chemical activity of the solute $\cong 1$
R = the gas constant = (0.00831 kg-mPa/mol-°Kelvin)
T = temperature in °Kelvin

In Marilyn's case, C in the above equation is 0.122 molal.

Fill in the rest and get Ψs:

Ψs = -(0.122)(1) (0.00831 kg-mPa/mol-°Kelvin)(293°K)

Ψs = -0.297 mPa

(iii) Finally, compute what the UNDILUTED Ψs value is for the leaf (before you added 3 mL of H_2O to grind it up in).

Marilyn added 3 mL H_2O to grind up her 0.23 gram of leaf. Since plant tissue is ~95% H_2O, we can assume that the leaf tissue contributed 0.23 mL of H_2O to the green puree. The total volume of puree is then 3.23 mL.

To obtain the true leaf Ψs, simply multiply the value in (ii) by 3.23 mL/.23 mL, the dilution correction factor:

$\Psi s_{(juice)}$ (dilution correction factor) = $\Psi s_{(leaf)}$
(-0.297 mPa)(3.23/.23) = (-0.297 mPa)(14.04) = - 4.17 mPa

Plant Physiology Fall 2011
Lab 6

End of lab directions

What you need to turn in today:

- **your completed data sheet**
- **The lab report for lab 4, leaf protein content.**

What you need to turn in next week:

Will I do a lab report for this lab, lab 6? : **Yes**, it will be due in two-weeks time on **Oct. 25.** I will tabulate the data as a class. We will use the collective class data for you to do your lab report for lab 6.

Appendix

2012 - HOW TO Plant Physiology Lab Experiments Report Format

1. Investigative question.

This is a question we are asking about the plant process or phenomenon that leads us to set up our investigation?

Example: the sun vs shade grown leaf study.....our investigative question could be

"Are the leaves found in the shaded, low-light regions of a tree different with respect to their photosynthetic structure compared to light exposed leaves?

2. Hypothesis

Formulate a hypothesis about our question that is falsifiable/testable by experimentation (H_1) **–** This begins with a theory you may have on what is happening BASED on previous knowledge (this previous knowledge for our purposes can be meager but it must have some basis being scientifically truthful).

Example: Shade leaves adapt to low light by boosting chlorophyll levels and increasing QY (photosynthesis efficiency) in order to trap and make the most use of what little light they do receive when compared to light exposed leaves.

3. Experimental design.

Testing the hypothesis experimentally: the experimental design. *For purposes of this class, I will not require technical details. You need to include only those aspects of how your data was collected.*

This can be done by a couple of paragraphs or simply using "bullets" to list the sequence of steps you did to get your data:
-harvest 4 shaded leaves and 4 sun exposed leaves into container of water
-place in dark cabinet for 30 min
-remove from water, daub with paper towel
-use hand held digital chlorophyll content meter
-clamp on center underside of leaf and make measurement

4. Results.

This will consist of graphs or tables NOT BOTH. In most cases, when you are able to graph your data using a bar graph, it will not be necessary to include a table version of your data.

-use Excel, online calculators, hand held calculators to convert your data into

✓ mean
✓ standard error or standard deviation
✓ students t-tests;

-then plot data graphically using a bar graphs with error bars, labeled x and y axis, a brief figure legend.
(see example figure as a "template" for how to compose your plots

5. Conclusion

Begin this section by revisiting what you stated in your hypothesis and then reflect on in writing. Consider whether or not your findings (the data) support or refute the hypothesis? Are differences between the treated and control statistically valid? Use the p value to validate what you can or cannot conclude from your experiment. If your hypothesis was falsified by your experiment, provide an alternative theory that accounts for your findings.

Plant Physiology Lab – 2012
Check List For Figure Format – See example below as a template for your graph

_____Black and white printing-NO COLOR

_____Leave a space between columns on chart graphs (no merged columns

_____You need to have error bars on the data points/chart columns

_____labeling of y-axis -always start at the ZERO

_____You must have a y-axis label – with Units of measure -- see example graph below

_____You must have an x-axis label –directly below column-- see example graph below

_____You must have a SHORT title inside or above the graph– a short title or label

_____You must have a figure legend in the exact format as specified– Begin legend as "Figure 1". , as if you are going to have multiple figures in your report and this is your first figure, and then add a brief one or two sentence description of what the figure is about. End ALL figure legends by including "data definitions" such as: ....."values are the means +/- s.d., n=4, p = 0.05;"

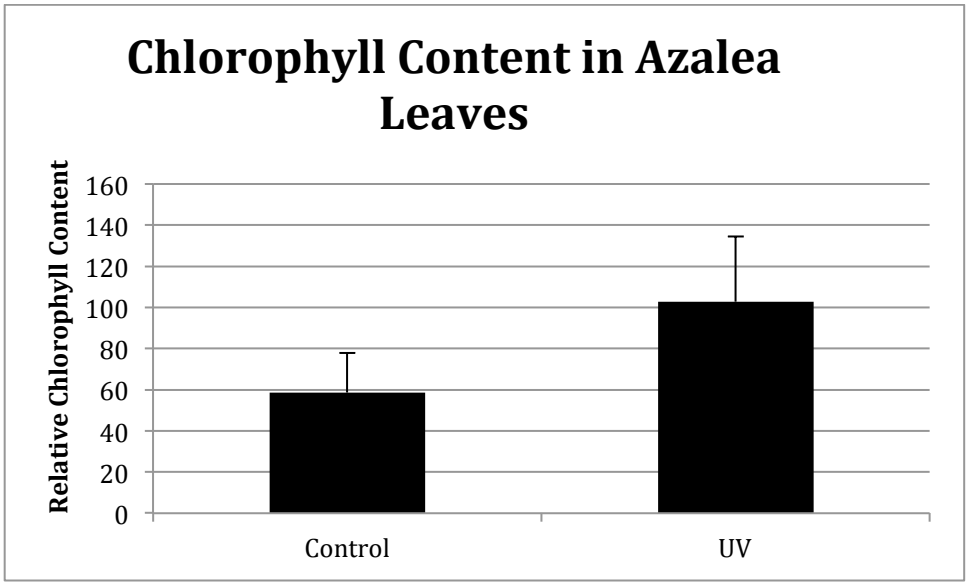


Figure 1. Chlorophyll content of UV treated and control leaves from azalea leaves. Values shown are the means ± s.d., control n=8, treatment n=8, p-value=0.019.

Examples of data plotted graphically in formatted figures with accompanying figure legends

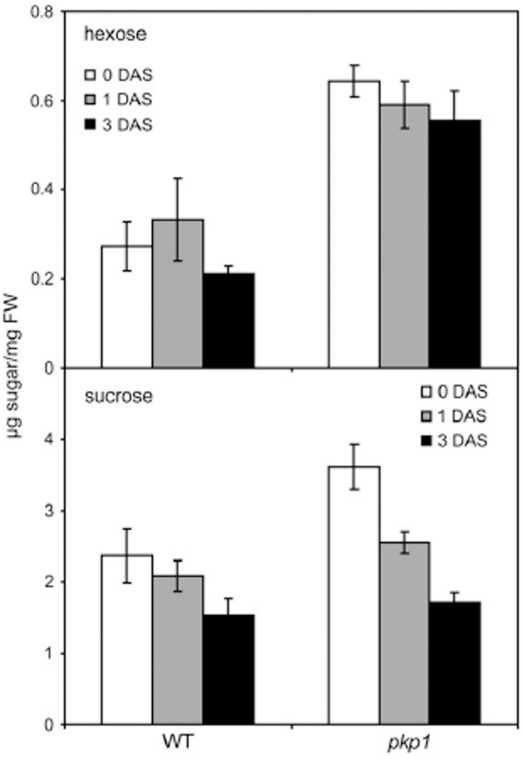


Figure 4. Soluble sugar amounts in the wild type (WT) and *pkp1* imbibed (0 DAS) and germinating seeds (1 and 3 DAS). Hexose (Glc and Fru) content is shown in the top panel and Suc content in the bottom panel.

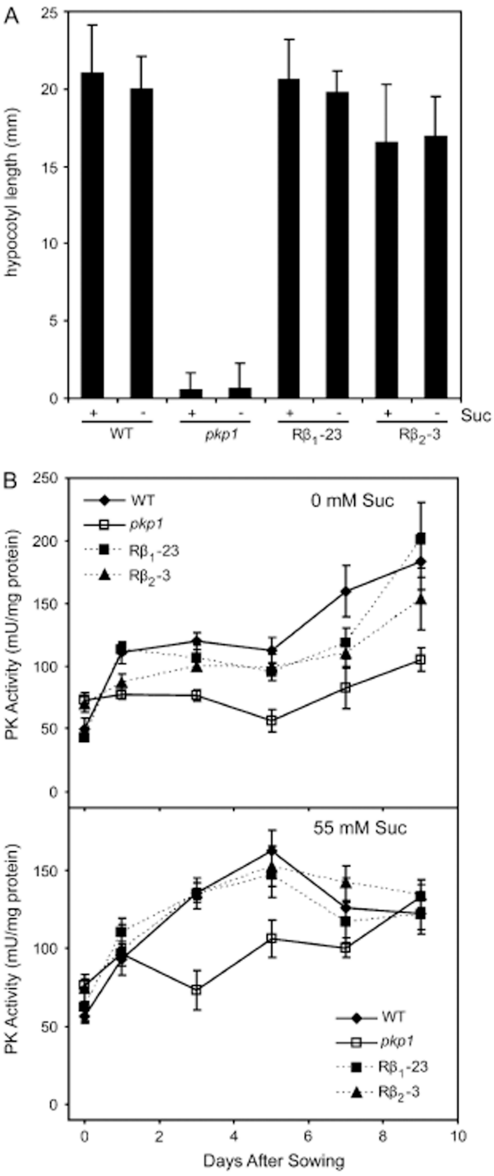


Figure 5. Hypocotyl elongation and PK activity in dark-grown seedlings. A, Length of hypocotyls 7 DAS of plants grown in the dark in the presence (+) or absence (−) of 55 mM Suc. Plant lines are as described in Figure 1. Values are the mean ± SD, $n = 25$. B, PK activity measured at pH 8.0 of etiolated seedlings grown with 0 mM (top) or 55 mM (bottom) Suc. Plant lines and symbols are as described in Figure 1. One milliunit is 1 nmol Pyr formed per minute. Values are the mean ± SD, $n = 4$.

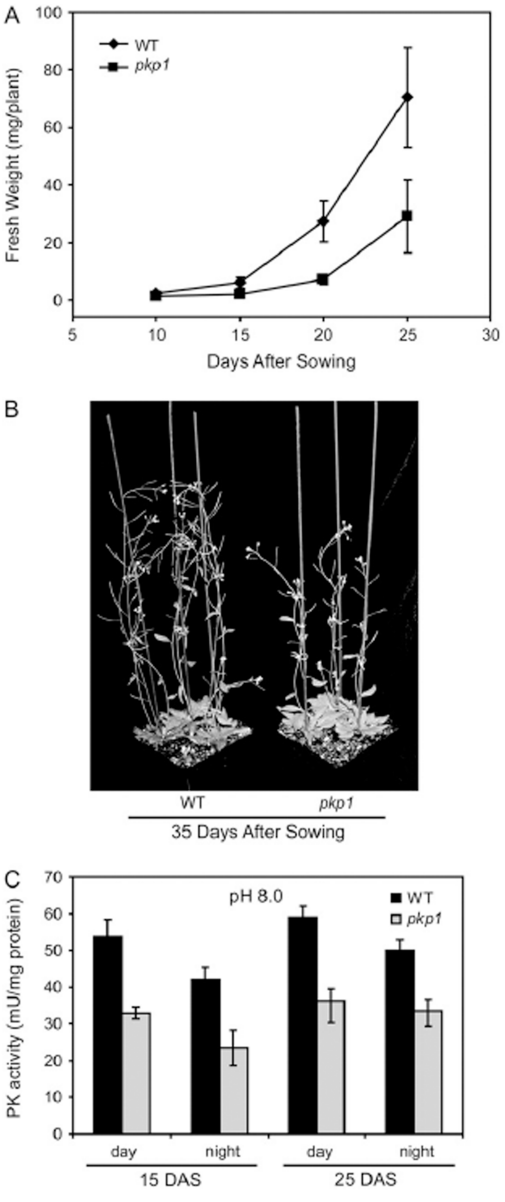


Figure 7. Tissue mass and PK activity in mature *pkp1* plants. A, Plant growth time course of plants germinated on Murashige and Skoog with 55 mM Suc and transferred to soil at 10 DAS. At each time point, aerial portions of six plants were excised and weighed. Values are the mean ± SD. B, Wild-type (WT) and *pkp1* plants 35 DAS. C, PK activity measured at pH 8.0. For each time point, five whole plants were homogenized and used to prepare protein extracts. Day = 8 h after lights on. Night = 8 h after lights off. One milliunit is 1 nmol Pyr formed per minute. Values are the mean ± SD (n = 4).

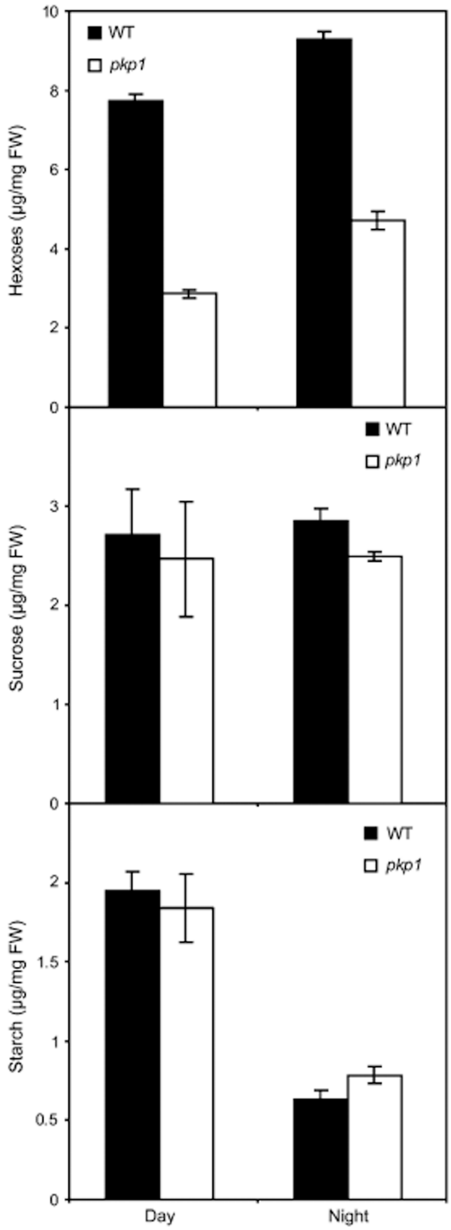
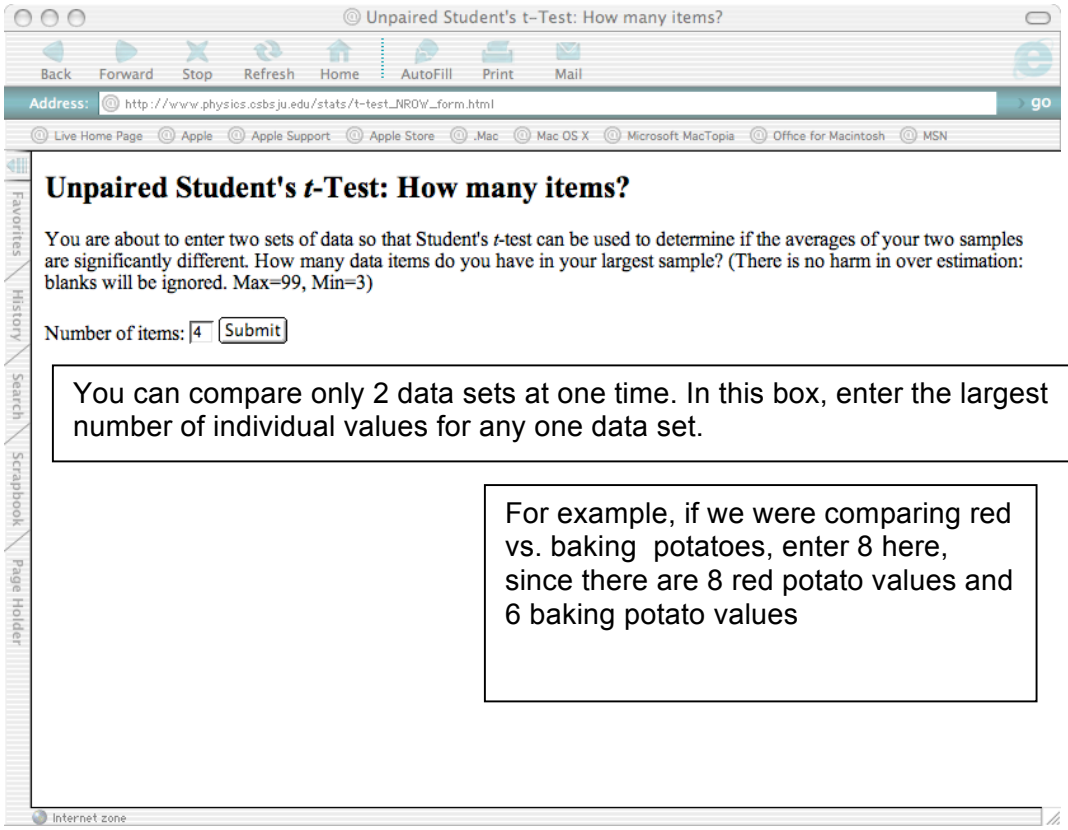


Figure 8. Carbohydrate content of 25-d-old leaves. Hexose (Glc and Fru, top), Suc (middle), and starch (bottom) content of rosette leaves is shown. Five entire plants were homogenized and used for extraction. Day/night cycle used was 16 h light and 8 h dark. Day = 8 h after lights on. Night = 8 h after lights off. Values are the mean ± SD (n = 4). FW, Fresh weight.

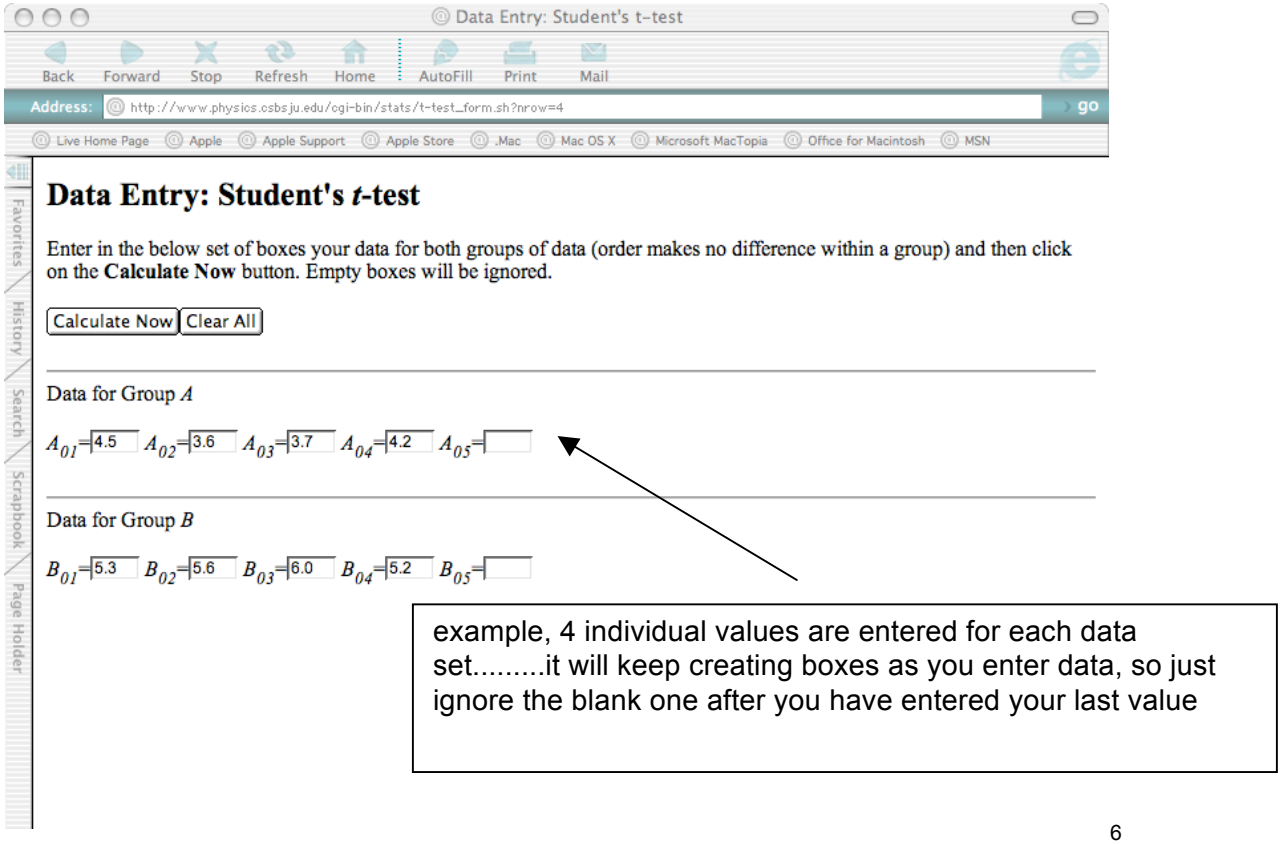
How to enter your data into the ONLINE-Calculator ------ Start Here:

1. go to: http://www.physics.csbsju.edu/stats/t-test_NROW_form.html

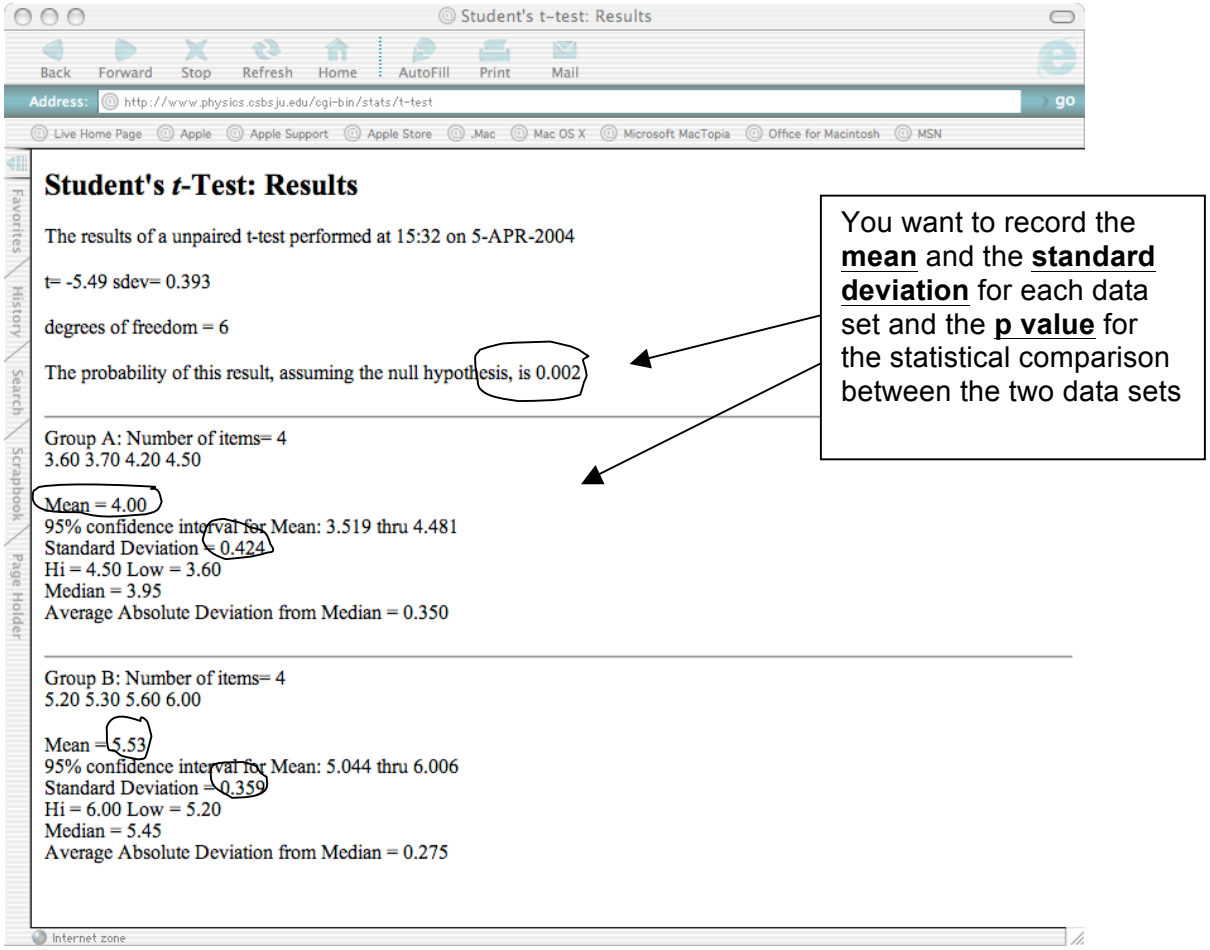
2. see this screen:



3. After submitting the number of data sets to be compared, enter the data individually for as many individual values you have.....



4. Finally, click on "CALCULATE NOW" and see the screen below.....this is your analysis



You want to record the **mean** and the **standard deviation** for each data set and the **p value** for the statistical comparison between the two data sets

Plant Physiology 2008

Intro to Digital Micro Pipettors a.ka. "Pipet Men"

For our lab, we will use three sizes :

20 μl

200 μl

1000 μl = 1 ml

1 μl = micro liter = 1 millionth of a liter = 1 x 10^{-6} liters

1 μl = 1 thousandth of a ml = 1 x 10^{-3} ml

What we use pipet men for:

(i) aliquoting

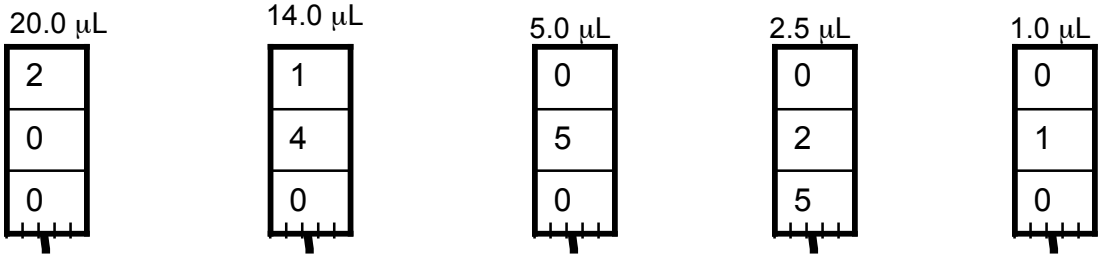
(ii) mixing/suspending - for small volumes

How to set pipetman for a given volume:

The confusing thing about using micropipettors is what you read on the dial or setting is different for each of the pipettors. For example, 100 on the on the 1000 μl pipettors is actually 1000 μl. And "020" on the 20 μl pipettor is not 20 μl but 2 μl. So the best way to learn how to set each one for a given volume is just to practice with them enough until it becomes intuitive.

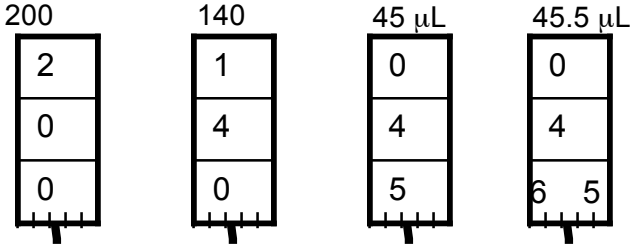
example settings **p20, 20 μl** pipettor

p 20



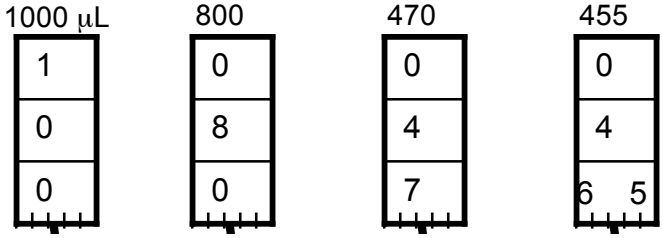
example settings **p200, 200 μl** pipettor

p 200



example settings **p1000, 1000 μl** pipettor

p 1000



Rules of thumb on how to choose the correct size pippetor to use for a given volume are:

-for pippeting volumes from 0.5 to 20 μl use the p20 μl pipettor

-for pippeting volumes from 21 to 200 μl use the p200 μl pippetor

-for pippeting volumes from 201 to 1000 μl use the p1000 μl pipettor

Don't, for example, use the 200 μl pipettor for 15 μl volumes.

Some other pointers:

1. When dipping your pipette tip into liquid to suck up fluid, just stick the tip a little below the surface. Don't plunge it way to the bottom. Otherwise, you will not get a very accurate volume.

2. When letting the button up to bring fluid into the pipet tip, let it up slowly and carefully. If you let it up to fast, then you end up sucking fluid into the pipettor.

3. When using the 200 or 1000 μl pipettor to mix or resuspend something, don't set the volume any higher than about a 150 μl. When you resuspend and suck up and down repetitively, the fluid will tends to creep up into the pipettor shaft.

4. When using the 200 or 1000 μl pipettor to mix or resuspend something, set the volume so it is less than the volume of what you are trying to mix. This prevents air bubbles and foaming in your sample.

Soluble Protein Content	
Leaf (μg protein/μL sap)	Xylem Sap (μg protein/μL sap)
12.7 +/- 2.1	0.063 +/- 0.011

Table 1. The text for your table legend should read the same as your figure legends you have been doing.