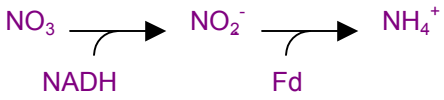
LEAF NITRATE REDUCTASE INDUCTION & ASSAY

Background: The nitrate assimilation pathway used by higher plants provides an excellent system for demonstrating control of gene expression in a eukaryotic organism. Assimilation of nitrate (NO_3^-) to ammonium (NH_4^+) takes place in a series of reactions:



The first reaction in leaf cells is catalyzed by a cytoplasmic enzyme nitrate reductase (NR). NR transfers two electrons from NADH to nitrate via the three redox centers composed of two prosthetic groups (FAD and heme) and a MoCo co-factor which is composed of molybdate and pterin. The complexity of NR is reflected in its size; it is a homodimer or homotetramer of 110 kDa subunits. The second reaction, the reduction of nitrite to ammonium is performed by a chloroplast enzyme nitrite reductase (NiR). A complete discussion of the nitrate assimilation pathway can be found in the plant biochemistry text on reserve for the course in the campus library.

Low levels of NR exist constitutively, but the majority of the enzyme activity is induced by exposure of plants to nitrate (Lewis, 1986). The rise in NR activity is due to *de novo* synthesis of NR, not activation of NR or translation of an existing mRNA for NR (Beevers, *et al.*, 1965; Sawhney & Naik, 1990). Light enhances the synthesis of both the nitrate-induced and constitutive NR activities (Lewis, 1986; Sawhney & Naik, 1990). However, light is apparently not directly involved in controlling synthesis of NR, but acts indirectly by influencing metabolic intermediates involved in the nitrate assimilation pathway (Crawford, 1995; Quesada, la Gomez-Garcia & Fernandez, 2000).

These experiments are modifications of exercises and procedures developed by Drs. Carl S. Pike (Franklin and Marshall College), Suzanne Harley (Weber State University), James Harper (University of Illinois) and Steven Huber (North Carolina State University).

References

Beevers, L., L. Schrader, D. Flesher and R. Hageman (1965) The role of light and nitrate in the induction of nitrate reductase in radish cotyledons and maize seedlings. *Plant Physiol..* **40**: 691-698.

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Harley, S. (1993) Use of a simple colorimetric assay to determine conditions for induction of nitrate reductase in plants. *The American Biology Teacher* **55**: 161-164.

Lewis, O. (1986) *Plants and Nitrogen*. Edward Arnold, London.

Quesada, A, la Gomez-Garcia and Fernandez, E. (2000) *Trends in Plant Science* **5**: 463-464**.**

Ross, C. (1974) *Plant Physiology Lab Manual.* Wadsworth, Belmont, CA,

Sawhney, S. & M. Naik (1990) Role of light in nitrate assimilation in plants. In *Nitrogen in Higher Plants.* Research Studies Press Ltd., Taunton, UK. pp 93-128.

NRI Lab 1: Growth of plants under various conditions of light and KNO_3

You will be provided with seeds that have been imbibed in water overnight. Plant these seeds and grow plants in vermiculite as follows:

1. Soak 2.5 g of radish seeds overnight in tap water (this has been done already for you).

2. Place a paper towel in the bottom of a 4" plastic pot. Moisten the towel with tap water.

3. Add dry vermiculite (inert mica medium) to within 1/2" of the top of the pot.

4. Decant water from imbibed seeds. Spread seeds evenly over the surface of the vermiculite.

5. Cover seeds with a thin layer of vermiculite.

6. Wet the surface liberally with base fertilizer, and allow to drain freely.

7. Grow the plants for one week under a 16 hr light/8 hr dark cycle at 20-25^{o}C in an environmental chamber watering with base fertilizer (0.1 mM $CaCl_2$, 0.1 :M Na_2MoO_4, 0.01% Fe-EDTA), with the following variations:

a. Base fertilizer (minus KNO_3) for 7 days (**light**)

b. Base fertilizer (minus KNO_3) for 6 days; fertilizer plus 20 mM KNO_3 for 24 additional hours (**light**)

c. Base fertilizer (minus KNO_3) for 6 days; **dark** and base fertilizer (minus KNO_3) for 24 additional hours

d. Base fertilizer (minus KNO_3) for 6 days; **dark** and fertilizer plus 20 mM KNO_3 for 24 additional hours

Base fertilizer: 0.1 mM $CaCl_2$
0.1 :M Na_2MoO_4
0.01% Fe-EDTA (sequestrene 330)

NRI Lab 2: *In vitro* and *in vivo* assays

IN VITRO ASSAY

Tissue extraction: At the beginning of the lab you should chill the necessary solutions, materials and equipment. Each group of students will prepare extract from one of the experimental treatments. See flow sheet (Fig. 1).

1. Remove green cotyledon leaves equivalent to 2 g. from the 7 day-old radish seedlings. To rapidly chill the tissue, place the leaves in ice-cold water. All subsequent operations must be carried out on ice.

2. Transfer the chilled leaves to a glass petri dish using a netting scoop, and finely slice the leaf tissue with a razor blade. Transfer the minced leaves to a cold mortar, and grind using 2 ml of Grinding buffer per g of leaf tissue.

Grinding buffer: 50 mM MOPS, pH 7.5
10 mM $MgCl_2$
1 mM EDTA
0.5 mg/ml DTT (dithiothreitol)
0.1 % Triton X-100

3. Strain the homogenate through one layer of miracloth into a 15-ml plastic tube on ice. Pour the filtrate into two 1.5 ml microfuge tubes, filling them to the top and discarding the remainder. Centrifuge at top speed in an Eppendorf microfuge for 10 min. at 4°C.

4. Transfer the supernatant liquid with a Pipetman pipette into a graduated tube, and store on ice. **This is your final extract sample**.

5. Draw 100 µl of your sample to a new microtube and store it at -20° for protein determinations during the next lab period.

Assay of Enzyme Activity: You will measure enzyme activities at three different volumes of extract (0, 15 & 50 µl), with and without the electron donor NADH. All activity assays are to be performed in duplicate!

***In vitro* assay solution**: 25 mM phosphate buffer, pH 7.5
10 mM KNO_3

NADH: 2.5 mM NADH in pH 7 buffer

6. Set up your enzyme reaction tubes and controls as follows:

a. Prepare duplicate 1.5 ml microfuge tubes, labelled “1" through “6" (*i.e.*, two tubes labelled “1", two tubes labelled “2", *etc.*)

b. Add 400 µl Standard Assay solution to each tube, then follow the table below :

Additions	Tube#1	Tube#2	Tube#3	Tube#4	Tube#5	Tube#6
Assay Med	400*	400	400	400	400	400
NADH	50	50	50	--	--	50
H_2O	--	--	--	50	50	--
Gr. Buffer	--	35	50	--	50	--
Extract	50	15	--	50	--	50
Zn Acetate	--	--	--	--	--	25**

* All volumes are in µl
** Add first

7. Place all of your tubes at 30°C for 5 min. to equilibrate them to the reaction temperature. Sequentially add extract at timed intervals (*e.g.,* 15-second intervals). You will want to stop each reaction (step 8) in that same sequence and interval, so that the incubation time for each is exactly 10 min.

8. Ten minutes after initiating each reaction, stop it by adding 25 µl of 0.1M zinc acetate. Stop each reaction sequentially in the same time interval that you used to initiate the reactions. A fine precipitate will form that must be eliminated. Centrifuge each microfuge tube for 5 min.at RT at 8000 RPM.

zinc acetate: 0.1 M ZnOAc in water

9. Transfer 475 µl of each supernatant with a Pipetman to a fresh 1.5 ml microfuge tube. Add 25 µl of PMS (to destroy excess NADH), mix and incubate in the dark for 10 min. at RT (put it in a cabinet below your bench). Phenazine methosulfate is light-sensitive; it should be handled quickly in subdued light and kept in the dark.

PMS: 1 mM phenazine methosulfate in water

Preparation of a Standard Curve: At the same time that you test for nitrite production in your experimental tubes, you need to calibrate the color reactions by preparing a standard curve using known amounts of nitrite. You will be given a stock solution of 500 nmol/ml (500 µM) KNO_2.

10. Make a series of at least five sequential 1:1 dilutions, using 500 µl of stock nitrite solution and 500 µl of distilled water at each step.

Nitrite stock: 500 µM KNO_2 in water

11. Transfer 500 µl from each dilution tube to a new 1.5 ml microfuge tube. Place 500 µl of distilled water in a microfuge tube to serve as a “zero nitrite control”.

Color Development and Spectrophotometry: You will use a "Color Development Reagent" to determine the amount of nitrite (enzyme product) in each reaction tube and each standard tube. Prepare just enough color reagent for all of your assay and standard curve tubes (500 :l per tube). The color reagent is somewhat unstable, and you should use a single batch for the entire experiment.

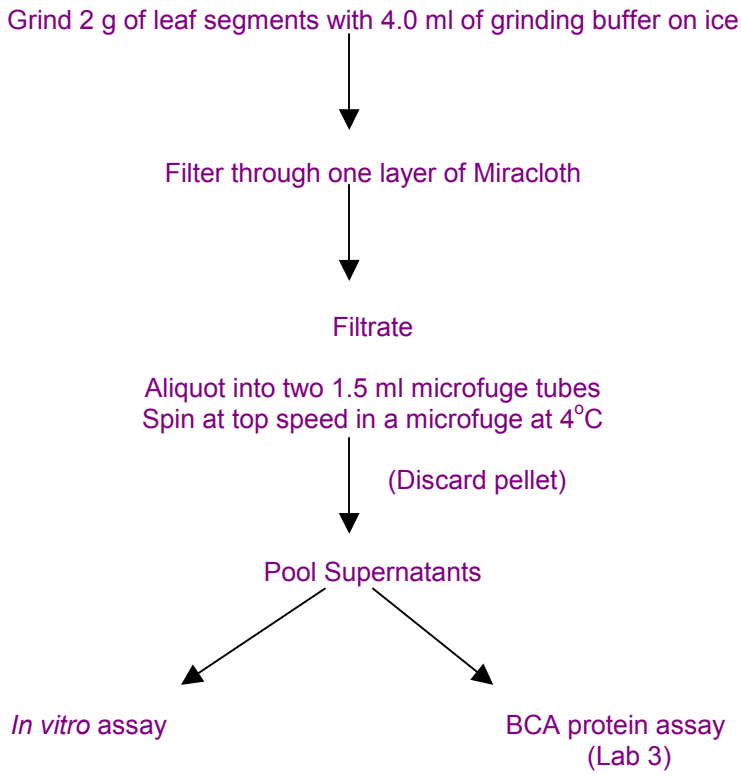
Color Development Reagent: 1% sulfanilamide in 1.5 N HCl
0.02% N-(1-naphthyl)-ethylenediaminehydrochloride

12. Immediately before use, combine equal amounts of 1% sulfanilamide (in 1.5N HCl) and 0.02% N-1(naphthyl)-ethylenediamine hydrochloride (in water).

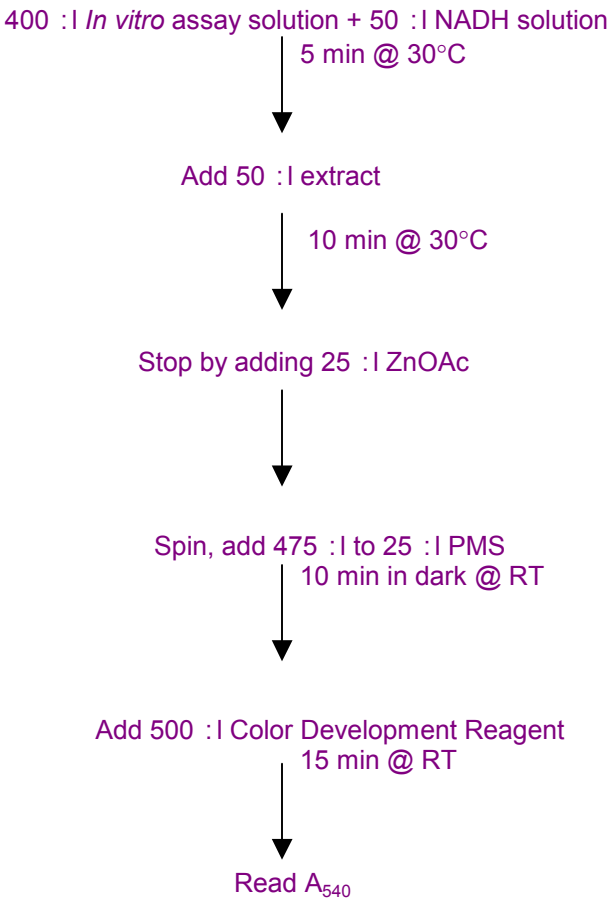
13. Add 500 :l of the color reagent to each reaction or standard curve tube, mix and incubate for 15 min at RT.

14. Zero the Pharmacia spectrophotometer at 540 nm using the "zero nitrite control". Measure the absorbance of all tubes, standards and experimentals. Average the values obtained for duplicates.

NRI Fig. 1: Flow Sheet for Preparation of Crude Leaf Extracts



NRI Fig. 2: Flow Sheet for *in vitro* Assay



IN VIVO ASSAY

Nitrate reductase (NR) activity can be directly assayed using cut leaf segments. In a solution containing nitrate, phosphate buffer and propanol, nitrate can readily enter leaf cells. In this case, the leaf cell supplies NR and the reductant NADH. The propanol in the assay medium increases cell permeability and makes the leaf segments essentially anaerobic. Under anaerobic conditions, the reduction of nitrite to ammonia is inhibited, and the conversion of nitrate to nitrite is enhanced Because of the sensitivity of the reaction to molecular oxygen all operations should be carried out in dim room light to reduce photosynthetic activity. With this approach the NR activity associated with all four inducing treatments can be assayed by one group.

1. Weigh out four 0.3g leaf samples for each of the inducing treatments: dark minus NO_3^-, dark plus NO_3^-, light minus NO_3^-, light plus NO_3^-. Cut the leaves into segments 1.0-1.5 cm squares.

2. Prepare duplicate 25 x 100 screw cap test tubes for each inducing condition. You will need one set for time=0 (t_o) and another set for time=30 min. (t_{30}). You should have a total of 16 tubes (4 conditions x 4 tubes).

3. Place the leaf samples in the appropriate tubes.

4. Add 10 ml of In vivo assay solution to each tube and cap the tube.

***In vivo* assay solution:** 100 mM phosphate buffer, pH 7.5
30 mM KNO_3
5%(v/v) propanol

5. Place the t_o tubes in a boiling water bath for 5 min. and then allow to cool to room temperature.

6. Place all tubes, t_o and $t_{30,}$ in a shaking water bath at 30^oC and incubate for 30 min.

7. At the end of the 30-min. incubation period, place the t_{30} tubes in a boiling water bath for 5 min. and then cool to room temperature.

Preparation of a Standard Curve: For the **in vivo assay,** you will also need a standard curve to calibrate the color reactions. You will be provided with a stock solution of nitrite.

Nitrite stock: 25 : M (nmol/ml) KNO_2 in water

8. Follow the table below to prepare the standard curve.

Tube #	nmol nitrite	ml nitrite	ml water
1	0	0	10
2	25	1	9
3	50	2	8
4	100	4	6
5	150	6	4
6	200	8	2
7	250	10	0

9. To detect nitrite in the assay tubes, add 10 ml of color development reagent.

Color Development Reagent: 1% sulfanilamide in 3N HCl
0.02% N-(1-naphthyl)-ethylenediaminehydrochloride

10. Mix thoroughly and place tubes in the dark at room temperature for 15 min.

11. Determine OD of each standard tube at 540 nm.

12. Add 10 ml of color reagent to each assay tube and determine the OD at 540 nm. Compare with standard curve.

NRI Lab 3: BCA protein assay

The bicinchoninic acid (BCA) assay for protein (Pierce Chemical Co. BCA Kit) is a derivative of the biuret reaction (protein reducing Cu^{2+} in an alkaline medium to produce Cu^{+}). Peptide bonds and four amino acids (cysteine, cystine, tryptophan and tyrosine) are reportedly responsible for this reduction reaction. Two molecules of BCA interact with one cuprous ion (Cu^{+}) to form a water soluble product that exhibits a strong absorbance at 562 nm. This allows the spectrophotometric quantitation of protein concentration in aqueous solution.

1. Prepare a set of eleven microtubes containing the following BSA protein standard dilutions (made from a 1 mg/ml BSA solution) and dilutions of your extract sample*.

Sample	Addition	Amount of Protein	A_{562}
A.	50 :l of H_2O	0 :g	
B.	5 :l BSA + 45 :l H_2O	5 :g	
C.	10 :l BSA + 40 :l H_2O	10 :g	
D.	15 :l BSA + 35 :l H_2O	15 :g	
E.	20 :l BSA + 30 :l H_2O	20 :g	
F.	25 :l BSA + 25 :l H_2O	25 :g	
G	50 :l BSA	50 :g	
H.	5 :l GB + 45 :l H_2O		
I.	1 :l extract + 4 :l GB + 45 :l H_2O		
J.	2.5 :l extract + 2.5 :l GB + 45 :l H_2O		
K.	5 :l extract + 45 :l H_2O		

* DTT in the Grinding Buffer will lead to color formation in the BCA assay, so equivalent volumes of GB must be maintained in each sample assay and a GB blank must be used.

2. Prepare Working Reagent by mixing 50 parts of Reagent A with 1 part Reagent B. The working reagent is stable for up to one day.

3. Pipet 1 ml of Working Reagent into each microtube, cap and mix.

4. Place the tubes at 37°C in a constant temperature water bath for 30 min.

5. Allow the tubes to cool to room temperature.

6. Measure the absorbance at 562 nm for each sample (B-K) in the spectrophotometer using the "zero protein sample" (sample A) as the reference.

7. Prepare a standard curve by plotting absorbance *versus* protein concentration of the standards.

8. Use the standard curve to determine the protein content of your undiluted leaf extracts (1.0, 2.5, 5.0 :l aliquots) .

Outline for Lab Report D: Nitrate Reductase Induction Studies

BIO 410

I. Introduction and Background (one-page limit)

Provide a brief description of our current understanding of the structure and expression of the nitrate reductase enzyme in plants.

II. Specific Aims

Concise list of the objectives of these experiments; can be a number list of single sentences.

III. Experimental Methods & Results

Describe what you did and present the results of those experiments. Experimental detail found in the manual need not be repeated. You will have to note your particular group's methods, however (*e.g.*, which treatments/experiments did you perform). Include figures and tables presenting your results (*e.g.*, graphs, tables).

1. Prepare standard curve plots by graphing the relationship between NO_2 concentration in nmol *and* A_{540} for both the in vivo and the in vitro cases.

2. Use the standard curve to determine the nmol of nitrite produced in each experimental(assay) tube. Remember to deduct the value obtained with the zero enzyme control!

3. Now express your results in conventional terms. First, express them as a standard rate (nmol nitrite formed per minute). Next, express the enzyme reaction rate per milligram protein used in the *in vitro* assay (specific activity), based on the results from your BCA protein assays. For the *in vivo* assay, express the enzyme reaction rate per gram of fresh weight per hour.

IV. Conclusions & Discussion (one and one-half page limit)

Self-explanatory. For the in vitro assays is there a requirement for extract and NADH? How do light and nitrate affect the levels of constitutive and inducible activities in leaves? Were comparable results obtained using the *in vitro* and *in vivo* methods of NR assay?

V. Recommendations for Course Improvement

Give us three recommendations of means by which these experiments or the course in general can be improved in the future. Do not repeat recommendations that you gave before.