# Chapter 10

# Induction of Nitrate Reductase in Plant Shoots

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## Contents



## Instructor Introduction

For the past four years we have done this exercise in a second year class in whole plant physiology. The exercise was adapted from an excellent paper by Harley (1993). The experiment seems to be fairly robust since it has never failed (although a few students may get 'bizarre' results). It demonstrates the phenomenon of enzyme induction by a substrate. Students see the time course of induction over 24 hours and the effect of inducer concentration on enzyme activity. Light is also required for the process and this too is examined. After the data are calculated and graphed, the occurrence of enzyme induction is easy to appreciate. The simplicity of the experiment makes this a very elegant demonstration of a complex biological event. Further background information is available in Lillo (1983) and Sivasankar and Oaks (1996).

The class performs three experiments in this exercise. Each pair of students does only one of these experiments and gets data from two other groups (who did different experiments) to prepare their lab report. Students are allowed to choose the other groups from which they obtain their data. They are told to select carefully, as it will be easier to write their lab reports and explain the results if they have "good data". This encourages them to examine the raw data of their colleagues, to think about what happened in the lab, and to think about what "good data" should look like. (How variable are the replicates? Do the values go up or down as expected?)

The exercise uses a single set of treated plants for the whole class which means that all the plants are harvested and divided up into samples by the class as a group. Then pairs of students pick up a set of samples from some of the pots to do their experiment. There are three parts to this experiment and three pairs of students could do the whole exercise and trade results. For this, you would only need 1.5 g of shoot tissue from each pot of plants. We usually have four pairs of students do the same experiment (replicates). For this you need about 5 g of shoot tissue per pot. Students seem to have trouble with preparing the samples as a group but then doing the experiment in pairs. To avoid students 'jumping the gun' and trying to begin their particular

experiment before all the samples are ready, make sure the dispensers containing the assay buffer are not available in the lab until it is clear that all the pots have harvested and the samples prepared and distributed. Be sure all the students are wearing safety glasses and gloves when handling the reagents. Everyone should wash their hands before leaving the lab.

Run a nitrite standard curve prior to the lab to ensure the reagents and spectrophotometers work properly. Keep the tubes to show the students what the color reaction looks like. Make sure all students save the solution as they measure the sample absorbance in the spectrophotometer. They should return the solutions to the sample test tubes until the instructor examines the data. (If the spectrophotometer is defective or used improperly, the students can then re-measure their samples.) When students collect data from other groups, they should record the standard curve data as well as the sample absorbances and fresh masses. If the spectrophotometers are not equivalent, they will not be able to use the standard curve prepared on their spectrophotometer to calculate nitrite concentration on samples measured with another instrument. (If the spectrophotometers are properly calibrated, all should give the same answer.)

We have a couple of computers in the lab running Microsoft Excel™. Students can enter their standard curve data, plot the graph, run a linear regression and print the results during the lab period. They may also start setting up a table to enter all the mass and absorbance information so they can simplify the calculations that need to be done for their report.

## Materials

For a lab section of 24 people (12 groups of two) (Quantity needed in parentheses)

**Equipment:**

Spectronic 20 (or equivalent, to read absorbance at 540 nm) (12)
Cuvettes (24)
Test tubes; 18 mm O.D. x 150 mm; (500 / lab) *Note*: Narrower 16 mm O.D. tubes become too full and difficult to mix.
Boiling water bath: to hold 170 tubes at once or a few smaller baths with similar total capacity
Wire test tube holders (6)
Thermometers (1 / bath)
Dispensers - 5.0 ml with 1 liter reservoir (6)
Dispensers - 10.0 ml with 1 liter reservoir (4)
Balances; semi-analytical, accurate to 1 mg (6 or more)
Vortex (6 or more)
Graduated pipets, 10 ml (24)
Test tube racks; 40 slots; (40):
14 for plant harvest, 24 for samples and standard curve; 5 for water baths
Erlenmeyer flasks: 125 ml (12), 50 ml (12)
Graduated cylinders: 50 ml (4)
Safety glasses (24)
Rubber Pasteur pipet bulbs (24)
Felt-tip glass markers, scissors, razor blades, distilled water bottles, spatulas, propipets: (12 of each)

**Supplies:**
Glassine weighing paper (24 sheets)
Plastic weighing boats; large (24)
Parafilm™ (2 rolls)
Pasteur pipets (14 cm long) (1 box)
Latex gloves (medium) (1 box)
Kimwipes™, Kleenex™, masking tape: (6 of each)

**Solution Preparation:**
pH meter, pH standards, acid/base solutions to prepare assay buffer.
Large volume vessels: 2, 4, and 10 liter plastic beakers or buckets, magnetic stirrer, balance.
Containers for +N fertilizer (e.g. 2-liter plastic 7-Up™ bottles, 6).
Bucket (10 liter) to prepare –N fertilizer on days 7, 10, 12 after seeds are started.
Bottles for nitrite solution (500 ml) (4)

**Chemicals:** (All reagent grade)
Distilled (Milli-Q) water (8 liter/lab for solutions)
$CaCl_2 \cdot 2H_2O$ (2 g)
Fe-EDTA (4 g): iron chelate (~13% Fe) from Plant Products Co. Ltd, Brampton, ON, Canada or Sequestrene (Ciba Geigy); something equivalent should be available from any horticultural dealer.
$NaMoO_4$ (50 mg)
$NaNO_2$ (50 mg)
NEED (500 mg) N-(1-napthyl)ethylenediamine dihydrochloride;
Sigma N5889; 10g – $ 34.50 CDN
HCl (concentrated) (500 ml)
Sulfanilamide (20 g) - Sigma S9251; 100g - $ 16.40 CDN
$KH_2PO_4$ (7 g)
$K_2HPO_4$ (44 g)
$KNO_3$ (25 g)
2-propanol (200 ml)

**Plants:** (for one lab section)
Corn seedlings: 14 pots per lab; any variety with good germination
~55 seeds / pot (1 tablespoon of corn is about 55 seeds; calibrate your supply)
(It is best to have a few more pots growing so one can select the 14 best; start 17 pots.)
Grow plants in *new clean* vermiculite or similar nutrient-free media
Clean pots: no nitrate/nitrite contamination; size ~ 15-20 cm diameter x 15 cm high
Clean drain pans under pots
If possible, grow plants in a controlled environment: 16 hour L (light):8 hour D (dark)
Lights on 0200 hours / off at 1800 hours;
*Note: lights on at 0200 hours is important to give 6 hours light before the morning lab.*
Temperature 23°C; ambient humidity in chamber
Relatively high light: >300 µE $m^{-2}s^{-1}$ Photon Flux Density
Age of plants: ~14 days; 3$^{rd}$ - 4$^{th}$ leaf expanding; shoots ~12 cm tall

You need ~5 g (fresh mass) of shoots / pot if each experiment will be replicated 4 times in the lab.
Water to saturation daily with Milli Q or distilled water.
-N fertilizer Solution: containing $CaCl_2$, Fe-EDTA and $NaMoO_4$ (see Appendix B, item 1A for details): fertilize on 3 dates, 500 ml / pot each time (~ days 7, 10, 12); total 7 liters for 14 pots each date; (21 liter total for all 3 days).
Pots can be placed together on clean trays until the day before the lab when nitrate is added. At that point, you must isolate treated pots using saucers under each to prevent any cross contamination of the roots in adjacent pots.
Clean pot saucers (drain trays) (14)
Dark cupboard to hold four pots, 2 days before the lab.
Fertilize with +N solution starting 24 hours before the lab period.

**Instructor's Notes**

**Safety Considerations:**

Use normal protective equipment for a 'wet' lab: lab coats, safety glasses, and latex gloves for those handling the sulfanilamide and NEED solutions and the samples after the reagents have been added.

N-(1-napthyl)ethylenediamine dihydrochloride (NEED)
CAS RN: 1465254
NIOSH #: KV 5330000
LD50 value ranks as "very toxic"
Negative carcinogenicity bioassay

Sulfanilamide:
CAS RN 63741
NIOSH #:WO 8400000
LD50 value ranks as slightly toxic
An experimental carcinogen.
*Note*: information gathered from Dangerous Properties of Industrial Materials by N.I. Sax, 6th ed. which also ranks human sperm as "an experimental carcinogen" so maybe that tells you something.

Both of these solutions are relatively dilute and are unlikely to pose a serious health hazard to students if they adopt good laboratory practices (no pipetting by mouth) and wear protective clothing. The biggest hazard in this lab is considered to be the 25% (volume/volume) solution of hydrochloric acid in the sulfanilamide reagent. Make sure students handling this reagent and the samples after the solution is added wear safety glasses and gloves. Wash off any spills with tap water.

**Pre-Lab Talk Notes:** (Some thoughts)

*Background:*

- Nitrogen is an essential element for plant growth.
- Most plants get nitrogen as nitrate from the soil.
- Nitrate is taken in through roots, then processed to a form that can be used in metabolism: $NO_3^-$ is reduced to $NH_4^+$ which is incorporated into other compounds (amino acids, amides, etc.).
- Nitrate reduction may occur in roots or shoots (plants differ).
- Enzymes are involved in reducing nitrate to ammonium; we will study one: nitrate reductase (NR).
- NR converts nitrate to nitrite.
- We can easily measure nitrite in samples. If we put in the substrate for the enzyme (nitrate) and see a lot of nitrite after a while (1 hour), then we can estimate the amount of enzyme activity present in the tissue.
- There may be nitrite in the tissue at the start so we measure the initial amount of nitrite, then 1 hour after nitrate is added, we measure it again. The net increase in nitrite at 1 hour is presumed due to NR activity.

*About enzymes:*

- Most enzymes are made up of peptides (chains of amino acids) that facilitate chemical reactions.
- Enzymes convert substrate(s) into product(s).
- DNA contains the information for making enzymes and other proteins. The DNA sequence specifies the mRNA sequence which determines the amino acid sequence, and this determines how the polypeptide folds and assembles into a catalytically active entity.
- Enzymes don't last forever; they are synthesized and destroyed over time. These reactions are probably regulated by other factors that depend on the environment inside/outside of cell or tissue. This is referred to as 'enzyme turnover'.
- Some enzymes may be present all the time (referred to as constitutive) while others are only present under certain conditions (inducible enzymes).
- Even after a peptide is synthesized, the enzyme may not be active or functional. The enzyme may consist of subunits (similar or different polypeptides) that need to be assembled. The enzyme might even require some non-protein cofactor such as a metal ion (e.g. Fe or Mo).
- In a multicellular organism, there can be different 'compartments' within a cell (e.g. cytoplasm, chloroplast, mitochondria, peroxisome, etc). These compartments may have different forms of an enzyme that catalyze the same overall reaction.
- A multicellular organism may have different tissues (specialized groups of cells with similar function, e.g. skin, heart, liver or leaf, root, seed). There may be different forms of enzymes in each tissue.
- The rate at which an enzyme converts substrate to product is referred to as the 'activity' of the enzyme. The rate depends on many things: concentration of substrates, products, cofactors, inhibitors, temperature and pH.

*About our experiment:*

- We will measure the activity of nitrate reductase in the shoot of a plant.
- Plants were grown without added nitrogen for 14 days; then, nitrate fertilizer was applied. The nitrate is expected to induce the enzyme after a time: little activity as soon as you add the nitrate and more activity later. We want to see the shape of the graph relating activity versus time since the fertilizer was added (Experiment A).
- In other experiments we keep the time constant (6 hours) but use different concentrations of nitrate in the fertilizer to see the relationship between activity and nitrate concentration (Experiment B). We also examine the effect of continuous darkness versus light/dark conditions during induction (Experiment C).
- Recall all the elements needed to make a functional enzyme. A system might not have an active enzyme simply because one element is missing or non-optimal (e.g. the polypeptide is all assembled in the organelle but the metal ion has yet to be inserted). Inducing the enzyme would simply consist of getting the metal ion into place. The situation with nitrate reductase is not this simple. Here induction by the substrate involves all the steps from DNA through peptide synthesis and assembly. This is a complex system that lends itself to further investigation at many different levels, either as a lab exercise or as an independent project.

*Practical Aspects:*

- Prepare the tissue from all the treatments. Label tubes, cut and weigh samples, and distribute tubes containing tissue to all the groups. Start the experiment by adding assay buffer.
- Don't cut the shoots until you have exclusive access to a balance. Ideally, one can process 20 tubes of tissue from a pot in about 15 minutes.
- Do not bring out the assay buffer until all the samples are prepared, assembled, and distributed to the class.
- *Caution*: Sulfanilamide dissolved in 25% HCl is corrosive. Wear gloves and eye protection.
- After the NEED solution is added to a tube, mix *immediately* by covering the tube with a strip of Parafilm™ and invert several times. Wait 20 minutes for the color to develop fully. The color is stable for hours.
- Have the teaching assistant look at the students' absorbance values before they discard any of their samples. If there is a problem, they can redo the measurements on another instrument.
- When students gather data from their colleagues for other parts of the experiment that they did not perform, be sure to get the standard curve values for the spectrophotometer used to measure the other samples.
- To avoid plugging the sink, filter leaf bits out of the test tubes when dumping solutions at the end of the lab. Use a piece of window screen over a funnel. One might collect all the solution waste in a bucket and neutralize the acid before pouring the solution down the drain. (Adjust pH in the 4 to 7 range by adding sodium carbonate.)

## Student Exercise

### Objectives

To observe the induction of an enzyme in plant tissue by studying:

a. The time course of induction,
b. The effect of substrate concentration on enzyme induction, and
c. The effect of light and dark on enzyme induction.

### Introduction

Nitrogen is a major element of plant tissues and occurs in many plant components including proteins (membranes, enzymes), nucleic acids and alkaloids. Certain plants can obtain nitrogen from the atmosphere via an association between microorganisms and the root system, but most plants take up either $NO_3^-$ or $NH_4^+$ from the soil. Due to the existence of conditions that favour oxidation of $NH_4^+$ to $NO_3^-$ by nitrifying bacteria in the soil, $NO_3^-$ is usually the predominant form of nitrogen entering many plants (see Salisbury and Ross, 1992).

In order for plants to make use of the nitrogen in biosynthesis, it must be in a more reduced state (i.e. as $NH_4^+$). To accomplish this, two enzymes are involved:

i) nitrate reductase (NR) catalyzes the reduction of $NO_3^-$ to $NO_2^-$. NR is a cytosolic enzyme (i.e. It is located in the cytosol of the cell rather than being contained within an organelle.):

**Equation 1** $NO_3^- + NADH + H^+ \rightarrow NO_2^- + NAD^+ + H_2O$

ii) nitrite reductase (NiR) catalyzes the reduction of nitrite to ammonium. NiR occurs inside organelles (chloroplasts or plastids):

**Equation 2** $NO_2^- + 6\ Fd(Fe^{2+}) + 8H^+ \rightarrow NH_4^+ + 6\ Fd(Fe^{3+}) + 2\ H_2O$

In chloroplasts, photosynthetic electron transport, driven by light energy, provides the electrons needed to reduce nitrite. Once formed, ammonium can be incorporated into carbon compounds by reacting with the amino acid glutamate to form glutamine. Glutamine can then pass on the nitrogen via a transamination reaction to many other carbon compounds.

Reduction of nitrate to nitrite via nitrate reductase may occur in both the plant root as well as the shoot, but in this experiment, we are only concerned with activity in the shoot. A very interesting feature of these two enzymes is that they are both inducible, which simply means that the presence of some chemical in the system causes the production of more enzyme. In this case, the inducer is nitrate, (the substrate for nitrate reductase), hence the process is an example of 'substrate induction'. One might speculate that this sort of enzyme induction is a way for an organism to conserve resources. Why bother making an enzyme if there is not substrate available with which to react? The lac-operon system is a well-known example of enzyme induction in microbial metabolism.

In this laboratory exercise, we will examine the induction of nitrate reductase by $NO_3^-$ in shoots of corn seedlings. Plants are grown in the absence of nitrate for about 14 days. Then a fertilizer solution containing $KNO_3$ is applied to the roots for a few hours before the activity of nitrate reductase in the shoots is measured. Three experiments will be conducted:

A. A time course of induction by $NO_3^-$ solution (20 mmol/liter):
Plants are provided with 20 mmol/liter $KNO_3$ at different times (0 - 24 hours) before the lab period. Shoot enzyme activity is measured.

B. The effect of $NO_3^-$ concentration on enzyme activity:
Plants are treated with different concentrations of $KNO_3$ (0 -100 mmol/liter) for a fixed time (6 hours) before the assay.

C. The effect of light on enzyme induction:
Plants that have been kept in the dark for 48 hours before the lab are compared with ones that had a normal light: dark cycle (16L:8D). Both types of plant are treated with either 0 or 20 mmol/liter $KNO_3$ for 6 hours before the lab.

**Experimental Protocols**

Enzyme activity is determined by placing leaf slices into an assay medium containing $KNO_3$ and measuring the accumulation of $NO_2^-$ over 60 minutes. We will measure the nitrite at the start of the assay (time 0 or $t_0$) and after 1 hour (time 60 or $t_{60}$) and calculate the rate of nitrite accumulation (i.e. the level of nitrate reductase activity) over this time. This procedure assumes that all the nitrite formed via nitrate reductase will accumulate in the sample and not be removed (e.g. by metabolism into $NH_4^+$ or beyond). To ensure that nitrite reductase is not active, keep the samples in dim light to prevent photosynthetic electron transport from supplying the electrons to ferrodoxin that are required for nitrite reductase. Also, NiR is inhibited by anaerobic conditions, so we add 2-propanol to our assay medium to waterlog the tissue and decrease the oxygen concentration. To measure the amount of nitrite in the samples, we employ a simple colorimetric reaction that converts nitrite to a purple azodye by reacting with sulfanilamide and 1-napthylethylenediamine HCl (NEED).

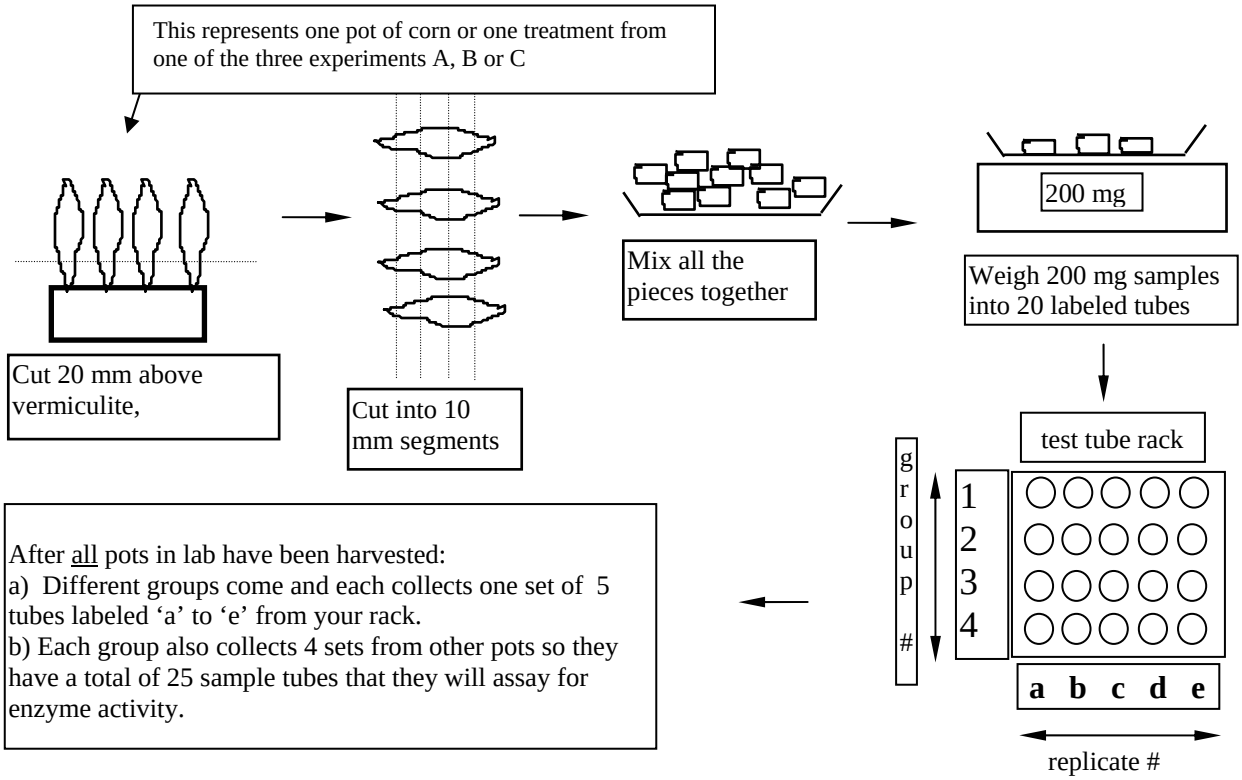
Each pair of students will be assigned one experiment (A, B or C) to do during the lab period. We pool the results from the whole lab for the report. We will have all the pots (5+5+4=14) of plants representing the 14 treatments that were prepared ahead of time. Due to the requirements of sample preparation, each pair of students will take one or two pots and prepare all the tissue samples required by the other groups in the lab for that treatment. After all the samples have been prepared, we will distribute them among the different groups. Pay close attention to the TA's instructions, since other students are depending on samples you prepare. Everyone must understand and use the same labelling system on the tubes.

A. *Sample Preparation:* (group effort)

**N.B.** Read and understand this entire section before proceeding with the tissue harvesting (See Figure 10.1).

Each group will harvest all the shoots from an individual pot (one treatment from Expt A, B or C). Cut the shoots about 2 cm above the vermiculite and remove any dead or yellowing leaves. Slice the leaves into 1 cm lengths with a single-edged razor blade. Mix all the slices together (randomize the sample), then weigh out 200 mg samples on glassine weighing paper and transfer the sample to a labeled test tube (18x150 mm). Record the exact mass of each sample on the worksheet provided. This harvest, slice, and transfer into tubes should be done quickly (~15 minutes) to minimize water loss by the tissue which will change the sample size during the weighing.

**Figure 10.1.** Representation of the harvest procedure for corn shoots.




Each treatment (pot of corn plants) will be used to prepare 20 tubes each containing about 200 mg of leaf tissue. Each tube will be labeled with:

1) The treatment:

For Experiment A: A/ time (hours) e.g. A/0, A/6, ... A/24

For Experiment B: B/ concentration of nitrate e.g. B/0, B/0.1, ...B/100

For Experiment C: C/ L or D, +N, or -N e.g. C/L+N, C/D+N, C/L-N, C/D-N

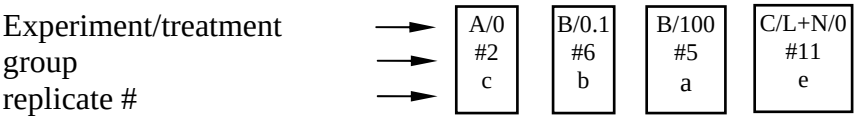
All 20 tubes prepared from one pot of corn will have the same treatment label.

2) A replicate number: a, b, c, d and e.

3) A group number: 1, 2, 3, 4 (for Part A), 5, 6, 7, 8 (Part B) or 9, 10, 11, 12 (Part C).

Four different groups of people will each take one set of five tubes (a,b,c,d,e) to conduct their experiment. They will also pick up four other sets of tubes (prepared from other pots of corn).

Here is how four of the labels from 4 different pots of corn should appear:




We will need to know the exact amount of tissue in each test tube so we will record the mass (mg) in a chart that will be provided in the lab (we will try to get all tubes with 190 - 210 mg of tissue). See Table 10.1.

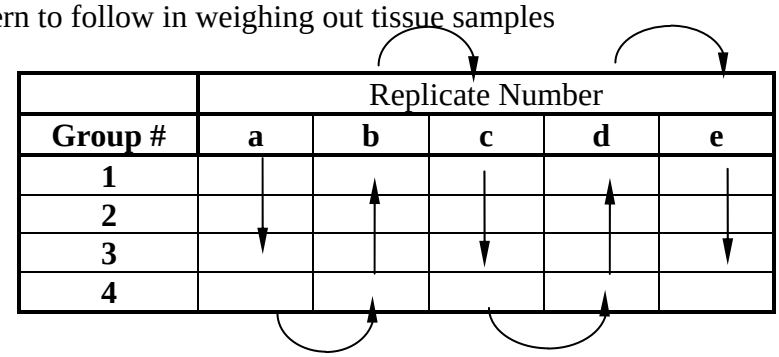
**Table 10.1**. Example of a chart to record the fresh mass (mg) of leaf tissue placed into each test tube from a pot of corn (one treatment).

**Experiment A: Time 6 hours**

| | Replicate Number | | | | |
|---|---|---|---|---|---|
| **Group #** | **a** | **b** | **c** | **d** | **e** |
| **1** | 201 | 207 | 195 | 210 | 198 |
| **2** | 206 | etc | etc | | |
| **3** | | | | | |
| **4** | | | | | |

To avoid errors, the 20 test tubes should be arranged in the rack in the same pattern as shown in the table. In order to randomize the samples during weighing, fill the test tubes in the order shown in Table 10.2.

**Table 10.2.** Pattern to follow in weighing out tissue samples



| | Replicate Number | | | | |
|---|---|---|---|---|---|
| **Group #** | **a** | **b** | **c** | **d** | **e** |
| **1** | | | | | |
| **2** | | | | | |
| **3** | | | | | |
| **4** | | | | | |

Note:

- Do not cut the shoots until you have access to a balance.
- Have all your test tubes arranged and labeled *before* you start harvesting the shoots.
- Weigh samples *quickly* to avoid water losses of the leaf slices.
- Fill the tubes in the sequence shown by your TA, again to randomize the variation among the samples. Fill all the 'a' tubes, then all the 'b's, etc.
- It should take about 15 minutes to fill 20 tubes.
- After all the tubes for the *whole lab* are filled, collect the samples you need to carry out your part of the exercise (part A, B or C).

**STOP HERE! Do not proceed any further.**

Once you have assembled all the samples required, start the assays. Later, get the individual mass data for your samples from the worksheets to use in your calculations of enzyme activity.

B. *Enzyme Assay Routine:* (Work in pairs; start after all pots of corn have been harvested and distributed)

Each sample tube contains about 200 mg of leaf tissue. There are five tubes (a, b, c, d, e) in a set of tubes. Tubes 'a' and 'b' are duplicates. Place these in a boiling water bath sample to kill the enzyme as soon as assay medium has been added to the (i.e. at time-zero). These tubes will give the amount of nitrite in the tissue at the start of the assay. After boiling 5 minutes, store the tubes at room temperature until the nitrite is measured. Add assay medium to tubes 'c, d, e' (triplicates) and incubate in the dark for 60 minutes before placing the tubes in the boiling water bath for 5 minutes. The nitrite measured in these tubes represents nitrite present at time zero plus nitrite produced during the 60 minutes by the enzyme nitrate reductase. We will subtract the amount of nitrite found in tubes 'a' and 'b' from that in tubes 'c, d', and 'e' to calculate the amount produced by enzyme activity during the assay period.

Experiment A: Time course of induction (five treatments: 0, 1, 3, 6 and 24 hours):

Five pots of plants were irrigated with a solution containing nitrate at different times before the lab period. Collect the 25 tubes (5 times x 5 tubes each time) of leaf samples from these five different pots. Start the reaction by adding 10 ml of assay medium into each tube and mixing. Use a glass rod to submerge the tissue. Tubes 'a' and 'b' are "killed" immediately (placed in the boiling water bath for 5 minutes). The other three tubes are placed in the dark at room

temperature for 60 minutes before killing. After all tubes have been killed and cooled, proceed with the nitrite assay procedure.

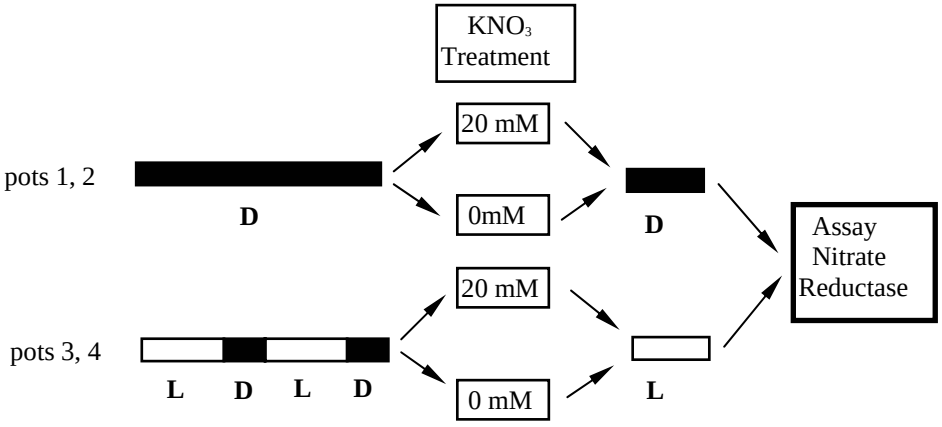
Experiment B: Effect of nitrate concentration on enzyme activity (Five treatments: 0, 0.1, 1.0, 10 and 100 mmol $NO_3^-$/liter):

Five pots have been treated with solutions containing different nitrate concentrations for 6 hours prior to the lab. Plants were kept in the light during the induction period. Collect 25 tubes (5 concentrations x 5 tubes of each concentration). Start the assay with 10 ml of assay buffer. Kill the time-0 samples (a, b) immediately. Incubate the 1 h samples (c, d, e) in a dark cupboard at room temperature for 60 minutes, then place the tubes in the boiling water bath for 5 minutes.

Experiment C: Effect of light and nitrate during induction (4 treatments):

Four pots of plants have been treated as depicted in Figure 10.2. Two pots (#1, #2) were placed in the darkness for 48 hours before the lab, while the other two (#3, #4) had a normal photoperiod (16L:8D). Six hours before the lab, one pot from the dark treatment (#1) and one from the light (#3) was treated with a solution containing 0 mmol/liter $KNO_3$. The other two pots (#2, #4) received 20 mmol/liter $KNO_3$.

**Figure 10.2.** Schematic of nitrate treatment for plants in Part C.




Pots were returned to their previous light regime for the 6-hour induction period. Collect the 20 tubes for this part. Label the tubes using the pot numbers shown in Figure 10.2. Start the assay by adding 10 ml of assay buffer. Boil the time-0 tubes (a, b). Incubate the time-60 samples (c, d, e) in a dark cupboard and boil the samples after 60 minutes.

C. *Nitrite Determination:*

When all the samples have cooled, add 5 ml (dispenser) of sulfanilamide solution to each tube. Mix with a Vortex, being careful not to spill the liquid.

Add 5 ml of NEED solution to a tube and *quickly mix the sample* by placing a strip of Parafilm™ over the mouth of the test tube, covering the tube with your thumb, and inverting the contents at least 5 times. Shift the piece of Parafilm™ along so a clean area covers the next tube. Return the tube to the rack and let them sit at least 20 minutes for the colour to develop before measuring the absorbance in the spectrophotometer at 540 nm.

Nitrite Standard Curve:

To quantify the amount of nitrite in the samples, prepare a standard curve by placing known amounts of nitrite in 10 ml of solution and treating them as you did the plant samples. Set up seven tubes labelled: 0, 1, 2, 4, 6, 8 and 10. Pipet the same volume (ml) of $KNO_2$ solution (25 µmol/liter) as labeled on the tube. Add distilled water to get a total of 10 ml in each tube (i.e. 10, 9, 8, 6, 4, 2, 0 ml). One milliliter of the $KNO_2^-$ solution contains 25 nmol so the standard curve ranges from 0 to 250 nmol $NO_2^-$.

Add 5 ml of sulfanilamide reagent to each tube and vortex. Add 5 ml of NEED solution and *mix each tube as you go* with Parafilm™ as described above. After sitting at least 20 min, measure the absorbance of each solution in the Spectronic 20. Start with the tube 0 and progress to tube 10 in sequence. Be sure to rinse the cuvette each time you change solutions. Do a final wipe of the cuvette with a slightly moist Kimwipe™ just before inserting it into the spectrophotometer. Be sure to orient the cuvette the same way for every sample. Rinse the cuvette with distilled water before doing the plant samples. Do all the time-0 tubes first (lightly colored: 'a, b') followed by the time-60 tubes ('c, d, e').

**Note:** After measuring the absorbance of each sample or standard, return the solution to its tube. Do not discard the solutions as waste until you show your results to your TA. If there were a problem with the spectrophotometer, you could still remeasure some of the tubes.

D. *Calculations:*

Plot the $A_{540}$ values for the standard curve versus the amount of nitrite (nmol) in the 10 ml sample volume. Determine the equation for the line by doing a linear regression (assuming there is a straight-line relationship). Include the zero point and obtain the regression coefficient ($r^2$) for the line. Using the graph directly, or better yet, using the equation from the linear regression, calculate the amount of nitrite in each sample tube. For each treatment, calculate the average amount of nitrite (nmol) for the two time-0 samples (a, b). Calculate the increase in nitrite for the three time-60 samples (c, d, e) using the average time-0 value. Calculate the rate of enzyme activity from the increase in nitrite over the actual period used (minutes) for the amount of each sample (fresh mass, FM) and express the rate as nmol nitrite $hour^{-1}$ $g^{-1}$ FM. Calculate the average rate for each treatment to plot the results, but also plot the individual values to give an idea of the variability in the data.

Because the samples were not all identical size to start with, we can reduce some of the variability by normalizing the nitrite levels to that of a 200 mg sample. For example Table 10.3 shows some sample data for one of the treatments.

**Table 10.3**. Sample data for calculating nitrate reductase activity

| Replicate | $NO_2^-$ measured (nmol) | Fresh Mass (mg) | $NO_2^-$ for 200 mg (nmol) | Assay time (minute) | $NO_2^-$ increase nmol/200mg | Nitrate Reductase nmol/60min /200mg | Nitrate Reductase nmol / hour /g FM |
|---|---|---|---|---|---|---|---|
| **a** | 10.0 | 190 | 10.5 | 0 | -- | -- | -- |
| **b** | 11.2 | 201 | 11.1 | 0 | -- | -- | -- |
| **c** | 147.5 | 203 | 145.3 | 64 | 134.5 | 126.1 | 631 |
| **d** | 163.8 | 208 | 157.5 | 64 | 146.7 | 137.5 | 688 |
| **e** | 202.1 | 213 | 189.7 | 64 | 178.9 | 167.7 | 839 |

For replicate 'a', the calculation is simply:

$$\frac{10.0\,\text{nmol}}{190\,\text{mg FM}} = \frac{X}{200\,\text{mg FM}}$$

Rearranging, $X = \dfrac{(10.0\ \text{nmol})(200\ \cancel{\text{mg}})}{190\ \cancel{\text{mg}}} = 10.5\ \text{nmol}\ NO_2^-$

For sample 'd': $\dfrac{163.8\ \text{nmol}}{208\ \text{mg}} = \dfrac{X\ \text{nmol}}{200\ \text{mg}}$

Rearranging, $X = \dfrac{(163.8\ \text{nmol})(200\ \cancel{\text{mg}})}{208\ \cancel{\text{mg}}} = 157.5\ \text{nmol}\ NO_2^-$

Take the average of replicates 'a' and 'b': ave = 10.8 nmol $NO_2^-$ at $time_0$ for 200 mg FM. Calculate the increase in 'c, d' and 'e' separately.

For 'e': 189.7 nmol - 10.8 nmol = 178.9 nmol in 64 minutes

Then: $\dfrac{178.9\ \text{nmol}}{64\,\text{min}} = \dfrac{X}{60\ \text{min}}$

and $X$ = 167.7 nmol $NO_2^-$ · 60 $minute^{-1}$ · 200 $mg^{-1}$ FM

For 1000 mg FM, $X = 167.7\,\dfrac{(1000\ \cancel{\text{mg}})}{(200\ \cancel{\text{mg}})} = 838.5\ \text{nmol}\ NO_2^-\ h^{-1}\ g^{-1}\ \text{FM}$

The average activity for replicates 'c', 'd' and 'e' is 719 $NO_2^-$ $hour^{-1}$ $g^{-1}$ FM. The range of 631 to 839 indicates some variability among these samples (which is why we do triplicates).

It is probably easiest to report your data in tabular format (Microsoft Excel™ is a spreadsheet program that is excellent for recording, calculating, and drawing graphs with data like this if you have access to this software). Be sure to show at least one example of each kind

of calculation you do to help us determine if you have correctly calculated the data. Plot data where it is appropriate (Experiments A and B). Be sure to label the axes correctly and to put a descriptive title on each graph.

Experiment A: Plot the relationship between induction time (hour) and enzyme activity. Prepare two graphs: one with a linear time scale and one with a logarithmic time scale. (It is probably easiest to use semi-log graph paper for this, but you can simply use a calculator to determine the logarithm of the time and plot these values on regular linear-linear graph paper.)

Experiment B: Plot the relationship between nitrate concentration and enzyme activity. Again do two graphs with a linear and a logarithmic x-axis.

Experiment C: Report results in tabular format.

E. *Analysis:*

Carefully examine all results and try to glean as much information as possible from the data. Look for internal consistencies (or inconsistencies) such as the results for the 6 hour treatment in Experiment A (20 mmol/liter $KNO_3$) and the Experiment C, pot #3 data for 20 mmol/liter $KNO_3$, induced for 6 hour in the light. Are the values the same? Do any further analysis necessary to interpret the results (e.g. make additional graphs, calculate percentages, etc.). What can you conclude about the induction of nitrate reductase in these plants from the observations you gathered here? Were there any problems in the experimental design that could be improved (e.g. what are sources of error or variability)? Do you have any suggestions for improving the experiment or modifying procedures to look at some other aspect of the phenomenon?

**Questions:**

1. Why plot the data in Experiments A and B on semilogarithmic paper?
2. How variable were the results in the $time_0$ and the $time_{60}$ samples (compare replicates)? Any suggestions how to reduce the variability (if you observed some)?
3. In this assay, we assume that the reaction of $NO_2^-$ to $NH_4^+$ was inhibited so we could get a good estimate of nitrate reductase activity simply by measuring the amount of nitrite accumulating during the assay. What could we do to test whether this was a reasonable assumption? If some nitrite was being metabolized further during the assay period, what effect would this have on the values of enzyme activity you calculated?
4. Describe how you might decide if any of the treatments used here were altering the activity of nitrate reductase in the roots. If a significant increase in activity in the roots was observed, would this have a bearing on the results we observed in the shoots? Explain.
5. Why do you think some enzymes are inducible and others are not? Do you think there is any significance to the fact that nitrate and nitrite reductase are substrate inducible enzymes?

**Literature Cited:**

1. Harley, S.M. 1993. Use of a simple colorimetric assay to demonstrate conditions for induction of nitrate reductase in plants. The American Biology Teacher 55(3):161-164.
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## Appendix A: Plant Growth and Nitrate Treatments

- For a lab section of 24 students (work in 12 pairs)
- Plants and solutions for 1 lab section:

### 1. Growth of Corn seedlings:

Prepare14 pots / lab section. Use 15 - 20 cm *clean* pots with about 55 seeds/pot. You need about 5 g fresh mass of shoot tissue from each pot (treatment). The amount of tissue needed depends on how many groups analyze that pot. (Typically four groups take samples from a single pot, i.e. the lab does four replicates of each experiment.) Plants are grown in vermiculite or other non-nutrient media for about 14 days at 16 hours L : 8 hours D, light about 300 μE $m^2$ $s^{-1}$, temperature about 23°C. Shoots have 3rd and 4th leaves expanding. Plants are about 10 cm tall. A controlled environment chamber is preferred over a greenhouse so the temperature can be better controlled to give a predictable plant size. Plants must not be exposed to any exogenous nitrate or nitrite during growth. Water daily to saturation with deionized water. A simple fertilizer solution is applied (500 ml/pot/day) after the seedlings start growing (about days 7, 10 and 12).

Germination: Fill pots with vermiculite (~90% full), spread seeds, and cover with 1 cm of vermiculite. Set pots on clean drain trays in a growth room and water with distilled water. Make sure pots are sitting in water at the start since dry vermiculite absorbs a lot of water. Water with distilled water to saturation daily.

### 2. Nitrate Treatment:

**2A**. Time course of induction: Five pots, 20 mM $KNO_3$; 24, 6, 3, 1, 0 hour exposure.
Place each pot in a clean saucer. Add 500 ml of the nutrient solution to one of the pots at different times before the lab. Make sure the liquid coming out the bottom of one pot does not contact another pot. (Hence the saucer under each pot).

**2B.** Effect of [nitrate]: Five pots; 6 hour exposure: 0, 0.1, 1, 10, 100mM $KNO_3$.
Place each pot in a clean saucer and add 500 ml of the nutrient solution to each of the pots 6 hours before the lab. Make sure the liquid coming out the bottom of one pot does not contact another pot.

**2C.** Effect of light on induction: Four pots, 0 or 20 mM $KNO_3$ at 6 hour before lab.
Follow the same procedure as outlined above with 20 mM $NO_3^-$. Two pots are placed in the darkness for a 48 hour prior to the lab. The other two remain in the growth chamber on a normal L:D cycle.

**Timing of Nitrate Application:**
Assume a morning lab at 0800 hours and afternoon lab at 1400 hours. The growth chamber is set for lights on at 0200 to allow 6 hours of illumination before the morning lab.

| | Morning lab | Afternoon lab |
|---|---|---|
| For Parts 2B and 2C, (6 hours before the lab) | 0200 | 0800 |
| For Part 2A: 24 hour († day *before* the lab) | 0800 † | 1400 † |
| 6 hour | 0200 | 0800 |
| 3 hour | 0500 | 1100 |
| 1 hour | 0700 | 1300 |
| 0 hour | 0800 | 1400 |

## Appendix B: Solutions and Equipment

- For a lab section of 24 students (work in 12 pairs)

**1. Solutions:**

**1A.** -N fertilizer solution:
Used during growth of the seedlings and to prepare +N fertilizer solution for inducing the enzyme. Prepare 100 ml of each stock solution. Mix 1 ml of each stock solution per liter of fertilizer solution. During growth, 14 pots require about 21 liters of -N fertilizer solution. Store stocks in the refrigerator. Prepare 7 liters of -N fertilizer as needed.

| Stock solution | Concentration | Amount per 100 ml | Stock volume (ml) for 7 liter of -N fertilizer |
|---|---|---|---|
| $CaCl_2 \cdot 2H_20$ | 0.1M | 1.46 g | 7 |
| Fe-EDTA (13.2% Fe) | 5g Fe/L | 3.79 g | 7 |
| $NaMoO_4$ | 0.1mM | 0.0024 g | 7 |

**1B.** +Nitrate Fertilizer for enzyme induction: (14 pots for 1 lab)

1B-1. Prepare 7 liters of -N fertilizer solution (7 ml of each stock in 7 liters distilled water).
Use 3.5 liters to make a 20 mM $KNO_3$ solution.
Use 3.5 liters to make the five other nitrate concentrations.

1B-2. $KNO_3$ stock: [1M] 15.2g / 150 ml distilled water

| **$[NO_3^-]$ (mM)** | **0** | **0.1** | **1.0** | **10** | **100** | **20** |
|---|---|---|---|---|---|---|
| [1M] $KNO_3$ (ml) | 0 | 0.05 | 0.5 | 5 | 50 | 70 |
| Total volume (liter) with –N fertilizer | 1.5 | 0.5 | 0.5 | 0.5 | 0.5 | 3.5 |

Solutions can be stored in clean 2-liter plastic soft-drink bottles. Store the solutions in darkness to avoid algal growth.

**1C.** Assay Buffer: For 12 groups, 280 test tubes to assay @ 10 ml/assay = 2800 ml.
Prepare 3000 ml / lab section.

| Reagent | Concentration | Amount for 3.0 liters |
|---|---|---|
| $KNO_3$ | 30 mM | 9.06 g |
| $KH_2PO_4$ | 100 mM | 6.54 g |
| $K_2HPO_4$ | 100 mM | 43.86 g |
| 2-propanol | 5% (v/v) | 150 ml |

Dissolve salts in 2.4 liter distilled water, adjust the pH to 7.5. Add the propanol, and bring to final volume. Provide 4 dispensers (750 ml/dispenser) set at 10.0 ml.

**1D.** Nitrite solution: 25 µM; prepare 500 ml / lab; used to prepare a standard curve.

1D-1. 10 mM $NaNO_2$ stock: 69 mg in 100 ml distilled water

1D-2. 25 µM $NaNO_2$: 5 ml of 10 mM $NaNO_2$ brought to 2000 ml total volume with distilled water.
Dispense four 500 ml bottles in lab.

**1E.** Nitrite Assay Reagents:

1E-1. Sulfanilamide solution: 1% (weight/volume) in 25% (volume/volume) HCl
Prepare fresh daily.
Use 5 ml / assay tube: 364 tubes/lab section = 1820 ml.
Make 2000 ml / lab section.
*Carefully* add 500 ml concentrated HCl to 1.5 liter distilled water. Dissolve 20 g sulfanilamide; bring the volume to 2.0 liters with distilled water.
Provide three dispensers set at 5.0 ml; (~ 670 ml / dispenser).

1E-2. NEED N-(1-napthy)ethelenediamine dihydrochloride solution: 0.02% (weight/volume) in distilled water.
Prepare fresh daily.
Use 5 ml /assay tube; 364 tubes/lab = 1820 ml.
Make 2000 ml / lab section.
Dissolve 400 mg NEED in 2.0 liter distilled water.
Provide three dispensers set at 5.0 ml; (~ 670 ml / dispenser).

## 2. Lab Equipment / Workstations:

**2A.** Plants:
Pots of corn seedlings (14) moved from the growth room just before lab starts.
Test tube racks, 40 slots (14).
Test tubes; 18mm O.D. x 150 mm long (300 / lab); for harvesting shoots.

**2B.** Tissue Harvesting stations: (6 in lab)
Place these stations on periphery of lab, away from where groups will do their assays.

Each station contains:
Plastic weighing boat; large (2)
Glassine weighing paper (2)
Semi-analytical balances; able to weigh samples to 1 mg
Scissors
Ruler
Forceps
Spatula
Single-edge razor blade

**2C.** Student stations: (12 / lab)
Test tube rack; 40 slot (2)
Test tubes; 18 mm O.D. x 150 mm, for standard curve (8)
Pasteur pipets; ~14 cm (4)
Rubber bulbs (2)
Distilled water wash bottle
Glass rod (to submerge leaves when add assay buffer)
Spectrophotometer (540nm)
Cuvettes (2)
Graduated pipets, 10 ml (2)
Propipet
Erlenmeyers: 125 and 50 ml
Marking pen

**2D.** Nitrate Reductase Assay:
Assay buffer; 3000 ml / lab in 4 dispensers; set 10.0 ml
Vortex (2)
Boiling water bath: one large (able to hold ~170 test tubes at once) or a few smaller ones
Thermometer(s)
Test tube racks; 40 slots (5)
Wire test tube tongs (6)

**2E.** Nitrite Assay reagents:
Nitrite standard: 25 µM; 2000 ml / lab in four bottles; 50 ml graduate for dispensing (4)
Sulfanilamide solution; 2000 ml / lab in 3 dispensers set at 5.0 ml

Vortex (2)
NEED solution: 2000 ml / lab in 3 dispensers set at 5.0 ml
Parafilm™ strips (~ 4x15 cm) (12)

**3. Miscellaneous Supplies:**

Latex gloves (24 pr)
Safety goggles (24)
Parafilm™ (2 rolls)
Kimwipes™ (6 boxes)
Kleenex™ (6 boxes)
Masking tape (6 rolls)

## Appendix C: Further Experiments

**Examine the assay system:**

1. Replicates often differ markedly. This has been noted before (Jaworski, 1971). What is the source of this variability? One might look at activity differences along the length of the leaf: compare the activity in 1 cm lengths from tip to base of a single leaf. (I recall a report discussing a gradient in RuBP carboxylase/oxygenase activity along a leaf blade). Maybe the variability is due to the assays system itself. Some leaf pieces are fully submerged while others are not. Can you force the tissue under with a piece of nylon screen? Does shaking tissue during the assay affect the variability?
2. Simplify the system: Can you measure length (or area) of leaves to prepare samples instead of using a semi-analytical balance to measure portions? How variable are the replicates when you try this?
3. Can you weigh all the samples from a pot and then freeze the tubes until the assay? This would allow you to prepare all the samples with a single balance a day or two (maybe more) before the lab. This would cut down the time required during the lab period to do the experiment.
4. The influence of oxygen on the assay is unclear both in terms of the response as well as the mechanism involved. It appears that the response depends on the assay conditions used (see Lillo, 1983). Is variability lower or enzyme activity significantly higher if you flush the samples with $N_2$ gas for a few minutes after assay buffer is added? (This may be impractical to do for a big class.) Does flushing the assay buffer with $N_2$ prior and during the lab give higher enzyme activity?
5. Can you test whether nitrite accumulation is a good estimate of nitrate reductase activity? Could nitrite be further metabolized in the tissue? What if you measured change in total amino acid levels (ninhydrin reaction) in the tissue? What if you measured change in individual products of nitrite assimilation like glutamine, perhaps by HPLC?
6. Could you develop a system that assesses nitrite reductase activity instead of nitrate reductase; supply nitrite instead of nitrate in the assay buffer and measure change in nitrite level over time? (You could use the same colorimetric assay or perhaps you could use a nitrite electrode.) Nitrite reductase is supposed to be inducible as well as NR. This might tell you if nitrite is being reduced during your assay for nitrate reductase (which complicates our assay for nitrate reductase).

**Investigate the plant system further:**

- Try shoots from different plants; some seeds (larger ones) may have bigger reserves of stored nitrate.
- Does this experiment work with algae? If so, it could be much easier to prepare samples with a pipettor than to use a balance to weigh 200 mg portions of shoot tissue.
- Examine how nitrate gets into the plant (the transporter system in roots) or examine factors that influence the transport of nitrate to the shoot (e.g. transpiration).
- Compare enzyme activity in the shoot versus the root. Compare this in different plants.
- How does light influence the induction process? Is the induction a photosynthetic response? What does the action spectrum look like (use colored filters)? Do photosynthetic inhibitors (herbicides like Divron or Simazine) alter the process? Does the $CO_2$ level affect the response? (Try to differentiate between a photosynthetic carbon product and an electron transport chain component). Is the phytochrome system involved? Are other photoreceptor system involved (e.g. blue-light effect)?
- Does a plant growth regulator like benzyl adenine affect the enzyme induction process?
- Nitrite and nitrite reductase are located in physically different cell compartments: Nitrate reductase is in the cell cytoplasm, while nitrite reductase occurs in the chloroplast of shoots (or plastid of root tissue). Are there any experiments you might try to investigate how the enzymes function in nitrogen assimilation? How does the nitrite get into the plastid for further reduction?

**Molecular aspects:**

- Measure mRNA production or peptide synthesis during the induction phase.
- Examine the effects of inhibitors of transcription or translation.
- Measure enzyme production using antibodies.

**Additional Reading:**

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## Appendix D: Data Sheets

### Part A: Fresh mass (mg) of leaf tissue

| Treatment | | | Group | Tube Number | | | | |
|---|---|---|---|---|---|---|---|---|
| Part | [nitrate] (mM) | Time (hours) | | **A** | **B** | **C** | **D** | **E** |
| A | 20 | 0 | 1 | | | | | |
| | | | 2 | | | | | |
| | | | 3 | | | | | |
| | | | 4 | | | | | |

| Treatment | | | Group | Tube Number | | | | |
|---|---|---|---|---|---|---|---|---|
| Part | [nitrate] (mM) | Time (hours) | | **A** | **B** | **C** | **D** | **E** |
| A | 20 | 1 | 1 | | | | | |
| | | | 2 | | | | | |
| | | | 3 | | | | | |
| | | | 4 | | | | | |

| Treatment | | | Group | Tube Number | | | | |
|---|---|---|---|---|---|---|---|---|
| Part | [nitrate] (mM) | Time (hours) | | **A** | **B** | **C** | **D** | **E** |
| A | 20 | 3 | 1 | | | | | |
| | | | 2 | | | | | |
| | | | 3 | | | | | |
| | | | 4 | | | | | |

| Treatment | | | Group | Tube Number | | | | |
|---|---|---|---|---|---|---|---|---|
| Part | [nitrate] (mM) | Time (hours) | | **A** | **B** | **C** | **D** | **E** |
| A | 20 | 6 | 1 | | | | | |
| | | | 2 | | | | | |
| | | | 3 | | | | | |
| | | | 4 | | | | | |

| Treatment | | | Group | Tube Number | | | | |
|---|---|---|---|---|---|---|---|---|
| Part | [nitrate] (mM) | Time (hours) | | **A** | **B** | **C** | **D** | **E** |
| A | 20 | 24 | 1 | | | | | |
| | | | 2 | | | | | |
| | | | 3 | | | | | |
| | | | 4 | | | | | |

**Part B: Fresh mass (mg) of leaf tissue**

| Treatment | | | Group | Tube Number | | | | |
|---|---|---|---|---|---|---|---|---|
| Part | [nitrate] (mM) | Time (hours) | | A | B | C | D | E |
| B | 0 | 6 | 5 | | | | | |
| | | | 6 | | | | | |
| | | | 7 | | | | | |
| | | | 8 | | | | | |

| Treatment | | | Group | Tube Number | | | | |
|---|---|---|---|---|---|---|---|---|
| Part | [nitrate] (mM) | Time (hours) | | A | B | C | D | E |
| B | 0.1 | 6 | 5 | | | | | |
| | | | 6 | | | | | |
| | | | 7 | | | | | |
| | | | 8 | | | | | |

| Treatment | | | Group | Tube Number | | | | |
|---|---|---|---|---|---|---|---|---|
| Part | [nitrate] (mM) | Time (hours) | | A | B | C | D | E |
| B | 1 | 6 | 5 | | | | | |
| | | | 6 | | | | | |
| | | | 7 | | | | | |
| | | | 8 | | | | | |

| Treatment | | | Group | Tube Number | | | | |
|---|---|---|---|---|---|---|---|---|
| Part | [nitrate] (mM) | Time (hours) | | A | B | C | D | E |
| B | 10 | 6 | 5 | | | | | |
| | | | 6 | | | | | |
| | | | 7 | | | | | |
| | | | 8 | | | | | |

| Treatment | | | Group | Tube Number | | | | |
|---|---|---|---|---|---|---|---|---|
| Part | [nitrate] (mM) | Time (hours) | | A | B | C | D | E |
| B | 100 | 6 | 5 | | | | | |
| | | | 6 | | | | | |
| | | | 7 | | | | | |
| | | | 8 | | | | | |

**Part C: Fresh mass (mg) of leaf tissue**

| Treatment | | | Group | Tube Number | | | | |
|---|---|---|---|---|---|---|---|---|
| Part | [nitrate] (mM) | Time (hours) | | A | B | C | D | E |
| C1 Dark | 0 | 6 | 9 | | | | | |
| | | | 10 | | | | | |
| | | | 11 | | | | | |
| | | | 12 | | | | | |

| Treatment | | | Group | Tube Number | | | | |
|---|---|---|---|---|---|---|---|---|
| Part | [nitrate] (mM) | Time (hours) | | A | B | C | D | E |
| C2 Dark | 20 | 6 | 9 | | | | | |
| | | | 10 | | | | | |
| | | | 11 | | | | | |
| | | | 12 | | | | | |

| Treatment | | | Group | Tube Number | | | | |
|---|---|---|---|---|---|---|---|---|
| Part | [nitrate] (mM) | Time (hours) | | A | B | C | D | E |
| C3 L/D | 0 | 6 | 9 | | | | | |
| | | | 10 | | | | | |
| | | | 11 | | | | | |
| | | | 12 | | | | | |

| Treatment | | | Group | Tube Number | | | | |
|---|---|---|---|---|---|---|---|---|
| Part | [nitrate] (mM) | Time (hours) | | A | B | C | D | E |
| C4 L/D | 20 | 6 | 9 | | | | | |
| | | | 10 | | | | | |
| | | | 11 | | | | | |
| | | | 12 | | | | | |

**Standard Curve for Nitrite** Spectrophotometer #: _____

[nitrite] solution = 25 µmol liter$^{-1}$

| Tube # | volume nitrite | volume water | Nitrite | A540 nm |
|---|---|---|---|---|
| | (ml) | (ml) | (nmol) | |
| 0 | 0 | 10 | 0 | |
| 1 | 1 | 9 | 25 | |
| 2 | 2 | 8 | 50 | |
| 4 | 4 | 6 | 100 | |
| 6 | 6 | 4 | 150 | |
| 8 | 8 | 2 | 200 | |
| 10 | 10 | 0 | 250 | |

**Part A:** Time course (0 - 24 hours) of enzyme induction after 20 mM nitrate

Tubes a, b = time 0 nitrite level
Tubes c, d, e = time 60 nitrite level

Data from Group # _____
Equation for nitrite standard curve:

| Time (hours) | Sample Tube | Assay Time (minutes) | A540 | Nitrite (nmol) | Fresh Mass (mg) | Nitrite (nmol / 200mg) | Average a,b (nmol / 200mg) | Net c,d,e (nmol / 200mg) | Average Activity (nmol / 200mg/hour) |
|---|---|---|---|---|---|---|---|---|---|
| 0 | a | 0 | | | | | | | |
| | b | 0 | | | | | | | |
| | c | 60 | | | | | | | |
| | d | 60 | | | | | | | |
| | e | 60 | | | | | | | |
| 1 | a | 0 | | | | | | | |
| | b | 0 | | | | | | | |
| | c | 60 | | | | | | | |
| | d | 60 | | | | | | | |
| | e | 60 | | | | | | | |
| 3 | a | 0 | | | | | | | |
| | b | 0 | | | | | | | |
| | c | 60 | | | | | | | |
| | d | 60 | | | | | | | |
| | e | 60 | | | | | | | |
| 6 | a | 0 | | | | | | | |
| | b | 0 | | | | | | | |
| | c | 60 | | | | | | | |
| | d | 60 | | | | | | | |
| | e | 60 | | | | | | | |
| 24 | a | 0 | | | | | | | |
| | b | 0 | | | | | | | |
| | c | 60 | | | | | | | |
| | d | 60 | | | | | | | |
| | e | 60 | | | | | | | |

**Part B:** Effect of nitrate concentration (0-100 mM) for 6 hour treatment

Tubes a, b = time 0 nitrite level
Tubes c, d, e = time 60 nitrite level

Data from Group # _____
Equation for nitrite standard curve:

| $[NO_3^-]$ (mM) | Sample Tube | Assay Time (minutes) | A540 | Nitrite (nmol) | Fresh Mass (mg) | Nitrite (nmol / 200mg) | Average a,b (nmol / 200mg) | Net c,d,e (nmol / 200mg) | Average Activity (nmol / 200mg/hour) |
|---|---|---|---|---|---|---|---|---|---|
| 0 | a | 0 | | | | | | | |
| | b | 0 | | | | | | | |
| | c | 60 | | | | | | | |
| | d | 60 | | | | | | | |
| | e | 60 | | | | | | | |
| 0.1 | a | 0 | | | | | | | |
| | b | 0 | | | | | | | |
| | c | 60 | | | | | | | |
| | d | 60 | | | | | | | |
| | e | 60 | | | | | | | |
| 1.0 | a | 0 | | | | | | | |
| | b | 0 | | | | | | | |
| | c | 60 | | | | | | | |
| | d | 60 | | | | | | | |
| | e | 60 | | | | | | | |
| 10 | a | 0 | | | | | | | |
| | b | 0 | | | | | | | |
| | c | 60 | | | | | | | |
| | d | 60 | | | | | | | |
| | e | 60 | | | | | | | |
| 100 | a | 0 | | | | | | | |
| | b | 0 | | | | | | | |
| | c | 60 | | | | | | | |
| | d | 60 | | | | | | | |
| | e | 60 | | | | | | | |

**Part C:** Effect of light and dark on enzyme induction after 0 or 20 mM nitrate

Tubes a, b = time 0 nitrite level
Tubes c, d, e = time 60 nitrite level

Data from Group # _____
Equation for nitrite standard curve:

| Treat-ment | Sample Tube | Assay Time (minutes) | A540 | Nitrite (nmol) | Fresh Mass (mg) | Nitrite (nmol / 200mg) | Average a,b (nmol / 200mg) | Net c,d,e (nmol / 200mg) | Average Activity (nmol / 200mg/hour) |
|---|---|---|---|---|---|---|---|---|---|
| C1 Dark 0 mM | a | 0 | | | | | | | |
| | b | 0 | | | | | | | |
| | c | 60 | | | | | | | |
| | d | 60 | | | | | | | |
| | e | 60 | | | | | | | |
| C2 Dark 20 mM | a | 0 | | | | | | | |
| | b | 0 | | | | | | | |
| | c | 60 | | | | | | | |
| | d | 60 | | | | | | | |
| | e | 60 | | | | | | | |
| C3 L/D 0 mM | a | 0 | | | | | | | |
| | b | 0 | | | | | | | |
| | c | 60 | | | | | | | |
| | d | 60 | | | | | | | |
| | e | 60 | | | | | | | |
| C4 L/D 20 mM | a | 0 | | | | | | | |
| | b | 0 | | | | | | | |
| | c | 60 | | | | | | | |
| | d | 60 | | | | | | | |
| | e | 60 | | | | | | | |