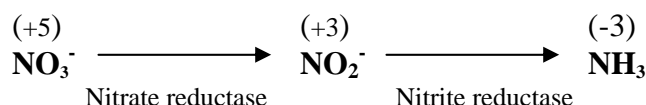


Activity of Nitrate Reductase in Plants

Effect of the availability and concentration of nitrate and light on the activity of nitrate reductase in Corn.

Plants obtain their nitrogen requirements mainly from the soil as nitrates. The fate of most of this nitrate is to be incorporated into proteins and nucleotides. This assimilatory nitrate reduction occurs via two enzyme-catalyzed reactions, which uses eight electrons and does not require ATP. Nitrate is first reduced to nitrite, which is subsequently reduced to ammonia. Ammonia is then incorporated into the amino acids glutamine/glutamate using the C-skeletons produced via other metabolic pathways such as respiration and photosynthesis.



The first reaction, reduction of nitrate to nitrite, is catalyzed by the metallo-protein nitrate reductase (NR) in the cytosol. Another metallo-protein, nitrite reductase, catalyzes the second reaction in the chloroplasts and /or plastids in which nitrite is reduced to ammonia. The electron donors for this sequential reduction is NAD(P)H (NR reaction) and reduced ferredoxin from PSII (nitrite reductase reaction).

Low levels of NR exists in plants constitutively, but most of the activity is induced by exposure of plants to nitrate (Lewis 1986). The molecular mechanisms that underpin the induction of NR activity are well characterized. Increased NR activity is due to *de novo* synthesis of mRNA and enzyme as well as to post-translational regulation by phosphorylation and dephosphorylation. (Beevers *et al* 1965, Sawhney & Naik 1990).

In this exercise we will investigate the conditions by which NR activity is induced in plants. This exercise demonstrates a simple colorimetric assay for determination of NR activity *in vivo* without the use of conventional enzyme extraction techniques (Harley 1993). Since NR is a highly unstable enzyme and the extraction of any enzyme requires considerable time this technique provides us with a simple way to determine NR activity in plant leaves.

During the assay leaf segments are incubated in a solution containing nitrate, 2-propanol and phosphate buffer (in Harley 1993). Nitrate enters the leaf segments, which supply both NR and reducing power. Nitrite is released into the assay medium and is converted to a bright magenta azo dye by reaction with Sulfanilimide and (SA) N-1-naphthylethylenediamine-HCl (NEED). If the nitrite level is to be measured the conversion of nitrite to ammonia must be stopped and the nitrite must be released from the cell. Under anaerobic conditions the reduction of nitrate is promoted and the reduction of nitrite is inhibited (Radin 1973). The presence of 2-propanol in the assay

medium increases the permeability of both nitrate into and nitrite out of the leaf segments (Kende *et al* 1971) and it provides an essentially anaerobic environment (Jaworski 1971). Because leaf tissue is used the experiment should be carried out under green light conditions to prevent the photosynthetic production of oxygen.

OBJECTIVES

To determine

1. the time required for induction of nitrate reductase
2. the effect of nitrate concentration on the level of nitrate reductase activity
3. the effect of light on levels of constitutive and induced nitrate reductase activity

You will carryout **one** of these three exercises. We will pool data from all groups and all exercises and post these data on on-line.

Exercise 1: Time course for induction of nitrate reductase in corn leaves

MATERIALS:

1. 7 – 14-day old corn plants grown in vermiculite, watered with mineral solution minus nitrate (Appendix 1). The treatment pots will receive 20 mM KNO_3^- at different time periods (see **Induction time** below) whereas the control pots will not receive nitrate (Table 1).

Table 1. Induction times for NR activity

<u>Tube #</u>	<u>Treatment</u>	<u>Induction time</u> (hours)
1	20 mM KNO_3^-	24
2	"	5
3	"	3
4	"	1
5	0 mM KNO_3^-	0

- 2 balance
- 3 boiling water on hotplates
- 4 30°C water bath (in the dark room)
- 5 5 ml and 10 ml pipets and bulbs
- 6 test tubes (with tightfitting stoppers) and racks
- 7 spectrophotometer

- 8 vortex mixer
- 9 Dark room with green safe light
- 10 pair of tongs
- 11 Sulfanilimide (SA) solution
- 12 N-1-naphthylethylenediamine-HCl (NEED) solution
- 13 Red & black marker pens

PROCEDURE:

1. Place 20 test tubes in a rack. Using a **black marker** pen (for **t = 0 h; this is the reaction time**) label **2 tubes** per treatment (1₀, 2₀, 3₀, 4₀, 5₀). This will result in **10** tubes labeled in black.
2. Label the remaining 10 tubes, (2 tubes per treatment) using a **red marker** pen (**t = 1 h; this is the reaction time**) 1₁, 2₁, 3₁, 4₁, 5₁. This will result in **10** tubes labeled in red.
3. Weigh out **0.3 g** of leaves for each induction time (x2 for duplicate tubes). Cut the leaves into segments of approximately 1 cm to 1.5 cm square. Place one set in t = 0 tubes (black) and the other set in t = 1 tubes (red).
4. Take the test tube rack into the dark room. **Turn off** the room light and **turn on** the green safe light.
5. Pipette 10 ml of assay medium (Appendix 2) into each tube. Cover the tubes.
6. Place the t = 1 tubes (red) in the water bath set at 30°C (in the dark room) and leave there for **1 h**.
7. Place the t = 0 (black) tubes in boiling water in beakers (What is the purpose of this step?). After **5 min** in boiling water remove these tubes and let cool at room temp.
8. While waiting for the 1 h reaction time construct the nitrite standard curve. Prepare tubes in duplicate according to Table 2.

Table 2. Volume of assay medium and nitrite solution needed to construct a nitrite standard curve (**make in duplicate**). The stock solution of nitrite contains 25 nmols/ml.

Tube #	Nitrite nmols	amount of Nitrite ml	amount of Assay Medium ml
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 each tube, add 5 ml of SA and 5 ml of NEED. Thoroughly mix the contents using a vortex mixer. Wait 10 min and read absorbance at 540 nm. (A_{540}).
10. Construct a standard curve by plotting A_{540} verses nmoles nitrite.
11. After 1 h incubation remove the $t = 1$ (marked red) tubes from the 30°C water bath and leave in boiling water for 5 min. Remove from the boiling beaker and leave at room temperature to cool.
12. In the meantime, carryout step 9 for $t = 0$ (marked black) tubes. When you read the A_{540} of the tubes use tube # 1 of the standard curve to zero the spectrophotometer
13. After cooling, add 5 ml of SA and 5 ml of NEED as in step 9 to the $t = 1$ tubes. Read the A_{540} of the tubes using tube # 1 of the standard curve tubes to re-zero the spectrophotometer.
14. Convert the A_{540} readings for the $t = 0$ and $t = 1$ samples from steps 12 and 13 to nmoles of nitrite using the standard curve.
15. Calculate the amount of NR activity in each treatment as nmols nitrite produced per hour per g fresh weight:

$$\text{nmols NO}_2^- \text{ h}^{-1} \text{ gfw}^{-1} = \frac{\text{nmols NO}_2^- (t=1) - \text{nmols NO}_2^- (t=0)}{0.3 \text{ g leaves}}$$

14. Plot NR activity against induction time in hours.
15. Discuss your results.

Exercise 2. The effect of nitrate concentration on the level of nitrate reductase activity

In this exercise you will determine the effect of different nitrate concentrations on the level of nitrate reductase activity. Planting and maintenance of the plants until the day of the experiment is the same as in exercise 1. The plants were not watered the day before the experiment. On the day of the experiment, 5 h (**9:30 a.m**) prior to harvesting, plants were watered according to Table 3 with 20 ml of nutrient solution containing 0, 0.1, 1, 10 or 100 mM KNO₃. Two pots were watered for each treatment. Five to six hours after watering the leaves are assayed for NR activity.

PROCEDURE:

1. Label test tubes in duplicate according to table 3.
2. You will harvest the leaves at 2:00 p.m (i.e 5h from treatment to harvest). While you are waiting prepare the standard curve. Follow steps 8-10 of exercise 1.

Table 3. Nitrate concentrations for induction of NR activity

<u>tube #</u>	[NO ₃ ⁻] (mM)
1	0.0
2	0.1
3	1.0
4	10.0
5	100.0

3. Follow the procedure as for exercise one and calculate the amount of nitrite produced per hour per g fresh weight of leaves.
4. Plot NR activity vs. nitrate concentration on semi-log paper. Discuss you results.

Exercise 3. The effect of light on levels of constitutive and induced nitrate reductase activity

The objective of this exercise is to determine the effect of light on inducible and constitutive NR activity. Plants were grown as for exercises 1 and 2.

1. **4 pots** were placed in the **dark for 48 hrs** (i.e. At 9:30 a.m two days prior to the lab).
2. After 48 h two pots from the dark treatment and two from the light were watered with nutrient solution **without nitrate** and the other two pots from the dark and light treatments were watered with nutrient solution **containing 20 mM nitrate**.
3. The dark-grown plants were returned to the dark.
4. After 5 h (i.e. 2:00 p.m), use the leaves for NR assay using the same procedure as in exercise 1.
5. Before you start label tubes in duplicate according to table 4.

Table 4. Treatments of light and nitrate for induction of NR activity.

tube #	TREATMENT	
	light	20 mM [NO_3^-]
1	yes	yes
2	yes	no
3	no	yes
4	no	no

6. Analyse and tabulate your results.

Appendix

Table 1. Stock solutions.

For +**NO₃**- growth medium **add KNO₃ separately** and **DO NOT add KCl** into those solutions.

NUTRIENT	ORIGINAL CONC.	AMOUNT/L	ELEMENTS	FINAL CONC.
CaCl ₂	100 mM	1 ml	Ca ²⁺ Cl ⁻	100 μM 100 μM
Fe-EDTA	5g/l	5 ml	Fe	25mg/l
Na ₂ MO ₄	0.1 mM	1 ml	Na ⁺ MO ₄ ²⁻	0.1 μM 0.1 μM
MgSO ₄	10 mM	1 ml	Mg ²⁺ SO ₄ ²⁻	10 μM 10 μM
NH ₄ H ₂ PO ₄	20 mM	1 ml	NH ₄ ⁺ PO ₄ ³⁻	20 μM 20 μM
MnSO ₄	100 μM	0.2 ml	Mn ²⁺ SO ₄ ²⁻	0.02 μM 0.02 μM
ZnSO ₄	100 μM	0.2 ml	Zn ²⁺ SO ₄ ²⁻	0.02 μM 0.02 μM
CuSO ₄	100 μM	0.05 ml	Cu ²⁺ SO ₄ ²⁻	0.005 μM 0.005 μM
*KCl	10 mM	10 ml	K⁺ Cl⁻	100 μM 100 μM
H ₃ BO ₃	2.5 mM	0.1 ml	BO ₃ ³⁻	0.25 μM

***1 M KNO₃ : 20 ml will be used only for the exercise 1 and exercise 3.**

For exercise 2 you have to refer to table 1 add the appropriate amounts of KNO₃.

Table 2. Composition of solutions to assay nitrate reductase (NR). All solutions should be prepared with dist. water.

E. Assay medium for NR: :	30 mM KNO ₃ 0.1 M K phosphate buffer (pH 7.5) 5% (v/v) isopropanol
To prepare E:	1.09 g of KH ₂ PO ₄ 7.31 g of K ₂ HPO ₄ dissolve in 400 ml of water. Add 1.51 g of KNO ₃ and dissolve. Add 25 ml of 2-propanol. Add dist water to make 500 ml. Check pH.
F. Reagents for nitrite assay:	
Sulfanilamide (SA)solution;	To 300 ml of dist. water, add 100 ml of Conc. (38%) HCl. Dissolve 4 g of SA in this solution.
G. <i>N</i>-1-naphthylethylenediamine dihydrochloride solution (NEED),	Dissolve 0.08 g (80 mg) NEED in 400 ml of dist. water.
H. Nitrite standard;	
10 mM nitrite stock solution:	Dissolve 69.00 mg of NaNO ₂ (or 85 .00 mg of KNO ₂) in 100 ml of dist.water. Prepare in a volumetric flask.
Working solution 25 nmoles NO₂ / ml:	Dilute, in a volumetric flask 1.00 ml of stock solution to 400 ml with assay medium. Keep in the fridge (4 °C)

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