Chapter 7

Nitrate reductase testing

Introduction

Although nitrogen is known as one of the most abundant biogens on Earth, a lack of this nutrient is a widespread phenomenon in many ecosystems. It is one of the most important factors limiting plant growth.
However, free nitrogen can be fixed from the atmosphere by certain prokaryotic organisms, both free-living and symbiotic. This form of nitrogen is not available to vascular plant species. It is available as nitrate and/or ammonia, which can be absorbed by tissues from the soil or atmospheric deposition.
Soil nitrate and ammonia are considered the most important source of nitrogen available to vascular plants, but gaseous pollutants such as nitrogen oxides, gaseous ammonia, nitric acid vapor, as well as nitrate falling with atmospheric dust directly absorbed by leaves can also influence the total reservoir of nitrogen available to plants.

Nitrate reductase (NR) is the enzyme that plays a key role in the nitrate fixation response to numerous environmental factors. Besides the presence of the substrate (nitrate), the enzyme's activity depends on many other factors such as temperature, plant water status, sunlight intensity, and others .

Due to the growing interest in biomonitoring and ecological studies, it has been necessary to develop new research methods. The research presented here attempted to find optimal conditions for the kinetic activity of enzymes using an in situ field experiment.

Definition

Nitrate reductase (NR) is also known as a soluble flavoprotein containing molybdenum. This enzyme is essential for the reduction of nitrate to NH_2 in plants, a crucial step in protein production. Most of the molybdenum (Mo) in plants is associated with this enzyme. Nitrate reductase protein synthesis is regulated by gene expression.
Nitrate reductase is the enzyme that catalyzes the first step of reducing nitrate N to organic forms in the plant, and it is thought to reflect the level of N activity in the leaves.

Nitrate reductase is a protein with an exceptionally short lifespan. Its half-life is only a few hours. The de novo synthesis rate of this enzyme is very high. Thus, by regulating its synthesis, nitrate reductase activity in tissue can be modified within a few hours.

Several factors control enzyme synthesis at the level of gene expression. Nitrates and light stimulate enzyme synthesis. Part of the light effect is caused by carbohydrates generated by photosynthesis. Nitrate reductase protein synthesis is stimulated by glucose and other carbohydrates generated by photosynthesis, and inhibited by NH_4^+, glutamine, and other amino acids. Sensors appear to be present in the cell that adjust, via the regulation of gene expression, the capacity of nitrate reductase to both the demand for amino acids and the supply of carbon skeletons derived from CO_2 assimilation for its synthesis.

By comparing leaf nitrate reductase activity with petiole nitrate concentration, it was found that nitrate reductase assays were a more sensitive and reliable indicator of plant nitrogen status. However, nitrate reductase assays are too expensive and time-consuming for routine use in assessing nitrogen levels in commercially grown cotton. Nevertheless, NR assays are useful for examining the effects of iron limitation on nitrate metabolism.

The objective of this research was:

- To develop a rapid, inexpensive, and reliable method for assessing nitrate reductase activity as a factor in the response to environmental stress.
- To determine the optimal conditions for the enzyme after the collection of plant material. The research was conducted in the Lipówka Forest Reserve, in the northern part of the Niepołomice Forest. This forest complex is located east of the Kraków metropolitan area. For many decades, it has been exposed to air pollution from Kraków, particularly from the steelworks (Mittel Steel SA, formerly known as the Nowa Huta Steelworks).

Materials and Methods

Five mature sessile oaks (Quercus sessilis) were selected for fresh leaf collection. To avoid water stress and minimize tissue damage immediately after collection, 3 mm diameter circles were cut using a hole punch and placed in test tubes (Fig. 1). Nitrate reductase (NR) activity is usually measured in vivo by assessing nitrite production in tissues infiltrated under vacuum with a buffered nitrate solution. For this study, a nitrate reductase assay was adapted from several studies with our own modifications. Because no power supply was available in this area, we used a hand-operated vacuum pump of our own design (Fig. 2).

Sampling and measurements were performed only in sunny conditions. The leaf tissue was then subjected to vacuum infiltration (using a hand-operated vacuum pump) at 0.33 atm for 5 minutes and incubated in buffer for 2 hours at 20 °C in the dark. The incubation buffer consisted of 0.1 M KNO_3 , 0.1 MkH_2HPO_4 , and 0.6% 1 - propanol , and was adjusted to pH 5.0, 6.0, 7.0, 8.0, and 9.0, respectively, using HCl and KOH.

The incubation temperature was set at 10, 20, 25, and 30 °C respectively. The temperature was adjusted and monitored using hot water or ice cubes to correct for fluctuations. The design of our incubation chambers allows for rapid correction of temperature changes if necessary (Fig. 3).

After incubation, the enzymatic activity was terminated by the addition of 1% sulfanilamide in 8% HCl. The concentration of synthesized nitrite in the incubation buffer was determined colorimetrically after diazotization and azo dye formation following the addition of the 0.02% N-(1-naphthyl) reaction mixture. diethylenediamine dihydrochloride.

Optical density was measured colorimetrically after 10 min at 540 nm using a spectrometer (Shimadzu UV-120). An incubation buffer mixture containing 1% sulfanilamide in 8% HCl and 0.02% N-(1-naphthyl)ethylenediamine dihydrochloride in the same proportions used to create the diazo compound was used as a blank. All chemicals were supplied by Merck (Germany).

The leaf samples were removed from the test tubes and weighed after oven drying until a constant weight was obtained at 60 °C. NR activity was calculated based on a calibration curve for KNO_2 . The
results were expressed in terms of the amount of nitrite synthesized in n mol per gram of dry weight of plant tissue per hour (nmol $g^{-1}DWh^{-1}$).

Fig. 1. Method for collecting leaf samples. Immediately after collecting the sample from the tree, circles of leaves were taken. This should be done as quickly as possible. If feasible, the circle should be taken without picking leaves from a single plant.

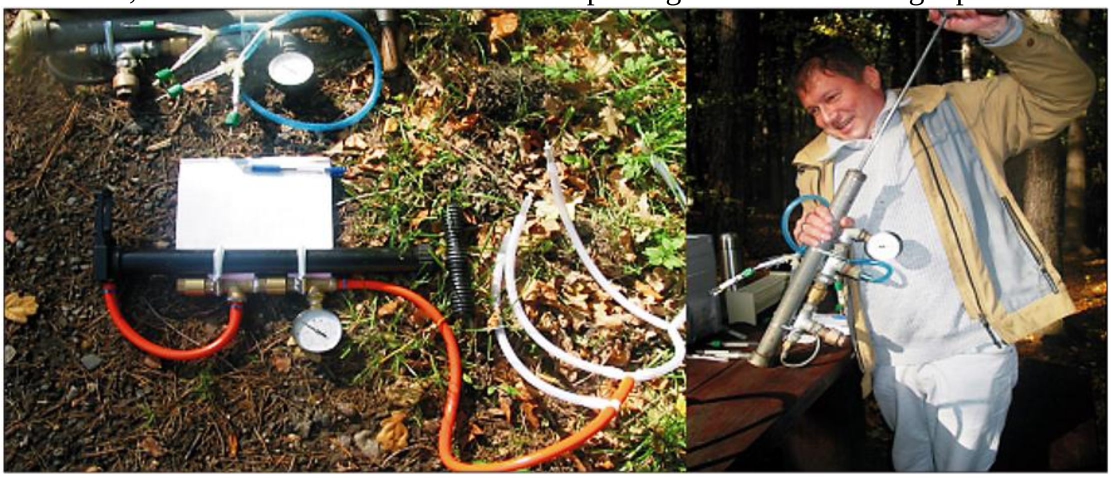

Fig. 2. After sampling, the leaf rings were placed in test tubes and subjected to low-pressure treatment (0.33 atm).
The manual low-pressure system consists of two valves, a pressure gauge, and a reverse piston pump.

Fig. 3. The temperature in the incubation chamber is stable; it is filled with water and allows for rapid temperature adjustment. The temperature is set and adjusted to a constant level throughout the incubation period using ice cubes or hot water.

Results and Discussion

Two factors influencing nitrate reductase activity were examined: the pH of the incubation buffer (experiment always performed at 20 °C) and the buffer temperature (always with the same buffer pH, fixed at 7.0). The results obtained showed no statistically significant difference in nitrate reductase activity with different buffer (pH) reactions ($F = 0.0011$, $p = 0.97$). However, it had, or tended to have, the highest activity between pH = 7 and pH = 8 (Mean = 314; EC = 39.6 nmol/g wt/h) (Fig. 4).

Probably no statistically significant difference in this study results from the small number of replicates. However, many authors indicate that this pH range provides an optimal environment for nitrate reductase activity. Therefore, the authors decided to use a buffer with a pH of 7.0 for the temperature incubation experiment. With temperatures ranging from 10 to 30 °C (M = 63; SD = 22.7; M = 490; SD = 283.6, respectively), a strong and significant correlation occurred ($r_s = 0.79$, $p = 0.00002$) (Fig. 5). However, many authors suggest using an incubation temperature between 20 and 25 °C to compare the results with other studies.

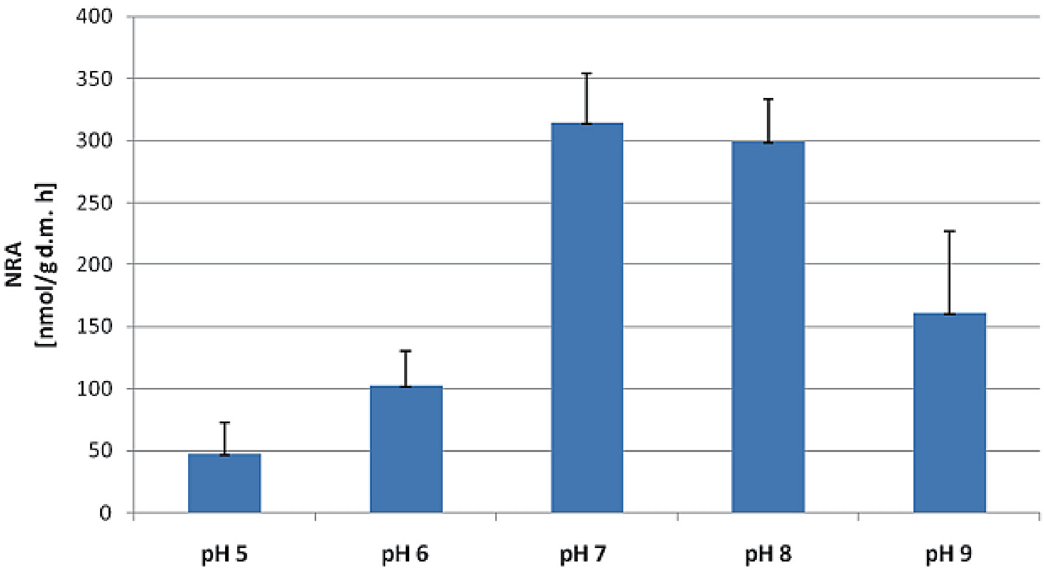

Fig. 4. Nitrate reductase activity is resistant over a wide pH range, but its optimum is between pH = 7 and pH = 8. However, the differences here were not significant, the trend is visible.

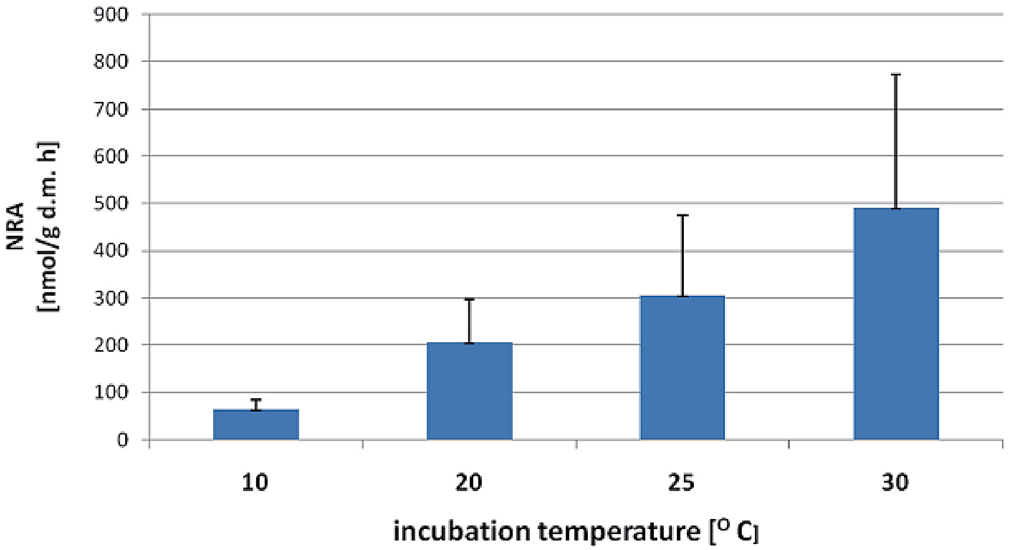

Fig. 5. Temperature has a strong impact on nitrate reductase activity. This impact is statistically significant (rs = 0.79, p = 0.00002) despite a high dispersion of results at the highest temperature studied (30 °C).

Conclusions

1. This research has demonstrated that nitrate reductase activity measured under field conditions can be a useful tool for studying plant growth. 2. Nitrate reductase exhibits resistance to changes in the pH of the incubation solution. 3. Nitrate reductase activity is strictly dependent on the temperature of the incubation solution.