Journal of Ecological Engineering
Volume 14, No. 1, Jan. 2013, pp. 7–11
DOI: 10.5604/2081139X.1031524

Research Article

METHOD OF MEASUREMENT OF NITRATE REDUCTASE ACTIVITY IN FIELD CONDITIONS

Marek Krywult[1], Dominika Bielec[2]

[1] Institute of Engineering and Environmental Sciences, Faculty of Materials and Environmental Sciences, University of Bielsko-Biala, 43-309 Bielsko-Biala, ul. Willowa 2, e-mail: mkrywult@ath.bielsko.pl

[2] Institute of Engineering and Environmental Sciences, Faculty of Materials and Environmental Sciences, University of Bielsko-Biala, 43-309 Bielsko-Biala, ul. Willowa 2, e-mail: dbielec@ath.bielsko.pl

Received: 2012.11.22
Accepted: 2012.12.13
Published: 2013.01.15

ABSTRACT

For the last three decades the interest in biomonitoring and ecological studies has been rapidly growing. Therefore, it was necessary develop of new methods of analysis for biochemical parameters which allow to quantify biological response of investigated organisms for environmental factors. The main goal of this paper demonstrates optimal conditions for enzyme kinetics analysis conducted in the field *in situ.*

Nitrate reductase activity is typically assayed *in vivo* by measuring nitrite production in tissue which has been vacuum infiltrated with buffered nitrate solution. For this study a nitrate reductase assay was adapted from a number of studies with own modifications of authors. Leaves of examined plants were collected from the investigated plots and immediately placed into test tubes with buffer solution (potassium phosphate dibasic containing 0.6% propanol-1) and evacuated in 0.33 atm. for 10 minutes. Then, known amount of potassium nitrate was added, and the solution sample was analyzed in order to obtain a background level of nitrite. The foliage samples were incubated for 2 hours at 20 °C in darkness. Following this procedure, they were given the most optimal conditions for reaction stability.

After incubation the amount of synthesized nitrite was determined colorimetrically using sulfanilamide and N-(1-naphthyl)ethylenediamine dihydrochloride, measured at 540 nm. The foliage samples were oven-dried to obtain dry mass. The level of nitrate reductase activity was calculated as the amount of nitrite produced in nmol per gram of dry mass of foliage tissue per hour. The result obtained during the research demonstrate the changes of nitrate reductase dynamics according to change of incubation parameters.

Dynamics of enzyme activity with changes of solution pH and incubation temperature was presented. Installation for conducting infiltration process and construction of incubation chamber is also described in this paper.

Keywords*:* nitrate reductase, kinetic changes, field analysis methodology, optimization.

INTRODUCTION

Although nitrogen is known as one of the most abundant biogens on Earth, lack of this nutrient is a widely known phenomenon in many ecosystems. It is one of the most important factors limiting plant growth [10, 11]. However, free nitrogen may be fixed from the atmosphere by some prokaryotic organisms, both free living and symbiotic [9, 15] this form of nitrogen is not available to vascular plant species. It is available in the form of nitrate and/or ammonia, which may be absorbed by tissues from soil or from atmospheric fallout [13, 14]. Soil nitrate and ammonia is believed be the most important source of available nitrogen for vascular plants, but gaseous

pollutants such as nitrogen oxides, gaseous ammonia, nitric acid vapor, also nitrate falling with atmospheric dust directly absorbed by leaves may also influenced the total pool of plant available nitrogen [5, 7, 16].

Nitrate reductase (NR), the enzyme which plays a key role in nitrate fixation response for many environmental factors. Besides substrate presence (nitrate), the enzyme activity is depended from many other factors such us temperature, plant water status, solar light intensity and others [12]. Because of growing of interest for biomonitoring and ecological studies, there was necessary to develop of new research methods. The presented research tried to find optimal conditions for enzyme kinetics activity with experiment conducted in the field *in situ.*

The aim of this research was:

- to develop fast, not expensive and reliable method to evaluable nitrate reductase activity, as one of factor response for environmental stress.
- to find of optimal conditions for the enzyme after collecting plant material

The research was conducted in Lipówka forest reserve in the northern part of Niepołomice forest. This forest complex is situated East from Cracow agglomeration. Since many decades it has exposed for atmospheric pollution from Cracow, especially from steel mill (Mittel Steel S.A., older called Nowa Huta Steel Mill).

MATERIALS AND METHODS

Five mature oak trees *Quercus sessilis* were chosen for collecting fresh leaves. To avoid water stress and minimize tissue damages immediately after collecting, circles of 3 mm diameter were cut using hole puncher and placed in the test tubes (Fig. 1). Nitrate reductase (NR) activity is typically assayed *in vivo* by measuring nitrite production in tissue that has been vacuum infiltrated with buffered nitrate solution [2]. For this study a nitrate reductase assay was adapted from a number of studies [1, 3, 13] with our own modifications [6, 8]. Because in this area no electrical power was supplied, we used manual vaccum pump of our own construction (Fig. 2). The sampling and measurements were carried out only on sunny days between the hours of 11 a.m. and 1 p.m. of the solar time.

The leaves tissue was then subjected to vacuum infiltration (with a manually operated vacuum pump) at 0.33 atm. for 5 minutes and incubated in the buffer for 2 hours at 20 °C in the dark. The composition of the incubation buffer was contained by 0.1M KNO_3, 0.1 M $K_2 HPO_4$ and 0.6% 1-propanol and adjusted to pH 5.0, 6.0, 7.0, 8.0 and 9.0 respectively, using HCL and KOH.

The temperature of incubation was set for 10, 20, 25 and 30 °C respectively. Temperature was set up and controlled with the use of hot water or ice cubes due to changes. Construction of our incubation chambers allows to fast correcting changes of temperature if it is necessary (Fig. 3).

After incubation the enzyme activity was terminated by the addition of 1% sulphanilamide in 8% HCl. The concentration of synthesized nitrite in the incubation buffer was determined colorimetrically upon diazotization and the formation of azo dye following the addition to the reaction mixture of 0.02% N-(1-naphthyl)

Fig. 1. Method of taking samples from leaves. Immediately after collecting the sample from the tree, circles of leave were taken. It should be done as fast as possible. If it is feasible, the circle should be taken without picking up leaves from a plan

Fig. 2. After sampling the leave rings were placed in test tubes and exposed to low pressure treatment (0.33 atm.). The manual low pressure system is constructed with two valves, vacuumeter and pump with conversed piston

Fig. 3. Temperature in incubation chamber is stable, it is filled with water and allow for fast adjust of temperature. The temperature is set and adjust to constant level for all incubation time using ice cubes or warm water

ethylenediamine dihydrochloride [3, 4]. Optical density was measured colorimetrically after 10 min. at 540 nm using a spectrometer (Shimadzu UV-120). A mixture of incubation buffer with 1% sulphanilamide in 8% HCl and 0.02% N-(1-naphthyl)ethylenediamine dihydrochloride in the same proportion as used in creating the diazo compound was used as a blank. All chemicals were supplied from Merck (Germany). The leaves samples were removed from the test tubes and weighted after oven-drying to a constant weight at 60 °C. NR activity was calculated on the basis of a calibration curve for KNO_2. The results were expressed as the amount of nitrite synthesized in nmol per gram of plant tissue dry weight per hour (nmol $g^{-1}DWh^{-1}$).

RESULTS AND DISCUSSION

Two factors influencing nitrate reductase activity were examined: pH of incubation buffer (experiment always conducted in 20 °C) and buffer temperature (always with the same buffer pH, set up on 7.0).

The obtained results demonstrate no statistical differences of nitrate reductase activity with different buffer reaction (pH) $F = 0.0011$, $p = 0.97$. But it has, or tends to the highest activity between pH = 7 to pH = 8 (AVG = 314; SD = 39,6 nmol/g d.m./h) (Fig. 4). Probably no statistical difference in this study results from small amount of replications. But many authors point to this range of pH as an optimal environment for nitrate reductase activity [2,12, 16]. Therefore, the authors decided to use buffer with pH = 7.0 for experiment with temperature incubation. With temperature ranged from 10 to 30 °C (AVG = 63; SD = 22,7; AVG = 490; SD=283,6 respectively), strong significant correlation occurred $rs = 0.79$, $p = 0.00002$ (Fig. 5). But many the authors suggest to use temperature of incubation between 20 and 25 degrees to compare the results with other studies.

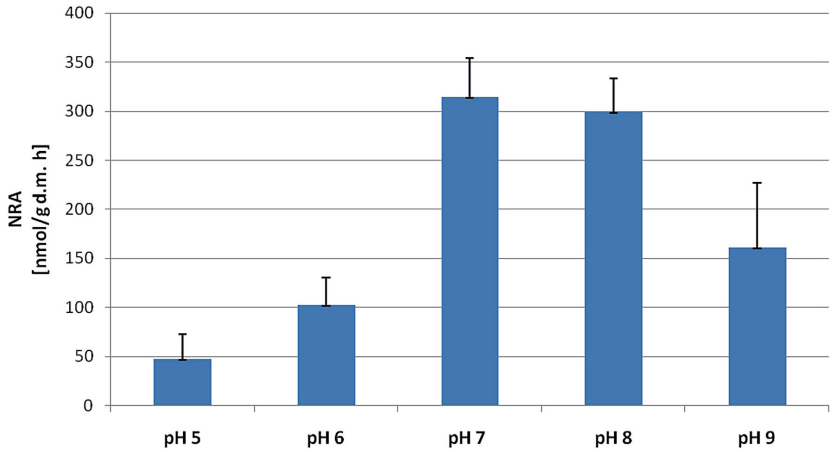

Fig. 4. Nitrate reductase activity is resistant with wide range of pH, but optimum of it placed between pH =7 and pH = 8. However differences here were not significant, trend is visible

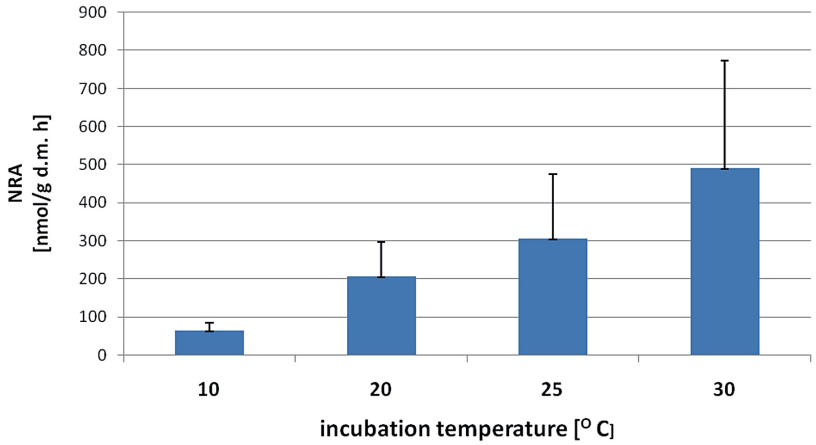

Fig. 5. Temperature have strong impact on nitrate reductase activity. It is statistically significant (rs = 0.79, p = 0.00002) in spite of high result dispersion in the highest investigated temperature (30 °C)

CONCLUSIONS

1. This research demonstrated that nitrate reductase activity measured in the field conditions may be a useful tool to study conditions of plants.
2. Nitrate reductase demonstrates resistance to changing pH of incubation solution.
3. Nitrate reductase for thr activity strictly depends on incubation solution temperature.

Acknowledgements

The authors would like to thank Dr. eng. Stanisław Braniewski from Institute of Botany, Polish Academy of Sciences in Cracow for logistical support and important and valuable rearks and advises, so as we would like to thank Dr. Damian Chmura for statistical analyses.

REFERENCES

1. Al Gharbi Q.A., Hipkin C.R. 1984. Studies on nitrate reductase on British angiosperms. New Phytologist, 97: 629-639.
2. Downs M.R., Nadelhoffer K.J., Melillo J.M., Aber J.D. 1993. Foliar and fine root nitrate reductase activity in seedlings of four forest tree species in relation to nitrogen availability. Trees, 7: 233-236.
3. Jaworski E.G. 1971. Nitrate reductase assay in intact plant tissue. Biochemical and Biophysical Research Communications, 43: 1274-1279.
4. Keeney D.R., Nelson D.W. 1982. Nitrogen – inorganic forms. [In:] Methods of soil analysis, Part 2. Chemical and biological properties. Ed. A.L. Page. Agronomy, 9, 2: 643-698.
5. Krywult M., Bytnerowicz A. 1997. Induction of nitrate reductase activity by nitric acid vapor in California black oak (*Quercus kelloggii*), canyon live oak (*Quercus chrysolepis*), and Ponderosa pine

(*Ponderosa pine*) seedlings. Canadian Journal of Forest Research, 27: 2101-2104.

6. Krywult M., Klich M. 2000. Nitrate reductase activity as an indicator of nitrate fixation and assimilation by tropical forest species on St. Thomas Island. Fragmenta Floristica et Geobotanica, 45, 1-2: 213-220.
7. Krywult M., Karolak A., Bytnerowicz A. 1996. Nitrate reductase activity as an indicator of Ponderosa pine (*Pinus ponderosa* Dougl. *ex.* Laws) response to atmospheric nitrogen deposition in the San Bernardino Mountains. Environmental Pollution, 93, 2: 141-146.
8. Krywult M., Turunen M., Sutinen M.-L., Derome K., Norokorpi Y. 2002. Nitrate reductase activity in some subarctic species and UV in influence in the foliage of *Betula pendula* Roth. seedlings. The Science of The Total Environment, 284, 1-3: 149-153.
9. Kumar A., Kumar H.D. 1988. Nitrogen-fixation by blue-green algae. [In:] Proceedings of the Plant Physiology Research, 1st International Congress of Plant Physiology. Ed. S.P. Sen. Society for Plant Physiology and Biochemistry, New Delhi, India: 85-103.
10. Mälkönen E. 1990. Estimation of nitrogen saturation on the basis of long-term fertilization experiments. Plant and Soil, 128: 75–82.
11. Nilsson L.O., Wiklund K. 1995. Nutrient balance and P, K, Ca, Mg, S and B accumulation in a Norway spruce stand following ammonium sulphate application, fertigation, irrigation, drought and N-free fertilisation. Plant and Soil, 168-169: 437-446.
12. Norby R.J. 1989. Foliar nitrate reductase: a marker for assimilation of atmospheric nitrogen oxides. [In:] Biologic markers of air – pollution stress and damage in forests. National Academy Press, Washington D.C: 245-250.
13. Norby R.J., Weerasurija Y., Hanson P.J. 1989. Induction of nitrate reductase activity in red spruce needles by NO_2 and HNO_3 vapor. Canadian Journal of Forest Research, 19: 889-896.
14. Pérez-Soba M., van der Eerden L.J.M. 1993. Nitrogen uptake in needles of Scots pine (*Pinus sylvestris* L.) when exposed to gaseous ammonia and ammonium fertilizer in the soil. Plant and Soil, 153: 231-242.
15. Sinha R.P., Kumar A. 1992. Screening of blue-green algae for biofertilizer. [In:] Proceedings of the National Seminar on Organic Farming. Ed. P.S. Patil. Pune, India: 95-97.
16. Wingsle G., Näsholm T., Lundmark T., Ericsson A. 1987. Induction of nitrate reductase in needles of Scots pine seedlings by NO_x and NO_3. Physiologia Plantarum, 70: 399-403.