Marek Krywult[1], Dominika Bielec[1]

MEASUREMENT OF NITRATE REDUCTASE ACTIVITY IN A FIELD CONDITIONS – METHODOLOGY

Abstract. During recent three decades interest for biomonitoring and ecological studies was rapidly growing. Therefore was necessary develop of new methods of analysis biochemical parameters whose allow 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 on 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. Follow this procedure have 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 their dry mass. 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 these 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 widely known phenomenon in many terrestrial ecosystems. It is one of the most important factors limiting plant growth [10, 11]. However free nitrogen

[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; e-mail: dbielec@ath.bielsko.pl

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 as to 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 the 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. Presented research tried to find optimal conditions for enzyme kinetics activity with experiment conducted in the field *in situ.*

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

STUDY AREA

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 of 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 were used manual vaccum pump 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

Fig. 1. Method of taking samples from the 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 circle should be taken without pick up leaves from a plant
Rys. 1. Metoda pobierania próbek z liści. Natychmiast po zebraniu liści z drzewa wycinano krążki tak szybko, jak to możliwe. W miarę możliwości krążki należy pobierać bez zrywania liści z drzew

Fig. 2. After sampling 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
Rys. 2. Wykrojone krążki liści umieszczano w probówkach i poddawano działaniu niskiego ciśnienia (0.33 atm.). System pompujący skonstruowano z dwóch zaworów, wakuometru oraz pompy z odwróconym tłokiem

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.

Temperature of incubation was set for 10, 20, 25 and 30 °C respectively. Temperature was set up and controlled using 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).

Fig. 3. Temperature in incubation chamber is stable, it is filled with water and allow for fast adjust of temperature. Temperature is set and adjust to constant level for all incubation time using ice cubes or warm water
Rys. 3. W komorze inkubacyjnej wypełnionej wodą utrzymywano stałą temperaturę za pomocą lodu lub gorącej wody

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)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 tender to have a tendency 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 this range of pH as optimal environment for nitrate reductase activity [2, 12, 16]. That`s why authors decided to put into use buffer with pH=7.0 for experiment with temperature incubation. With temperature ranged from 10 to 30^0C (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 Authors suggest to use temperature of incubation 20 of 25 degrees to may compare results with other studies.

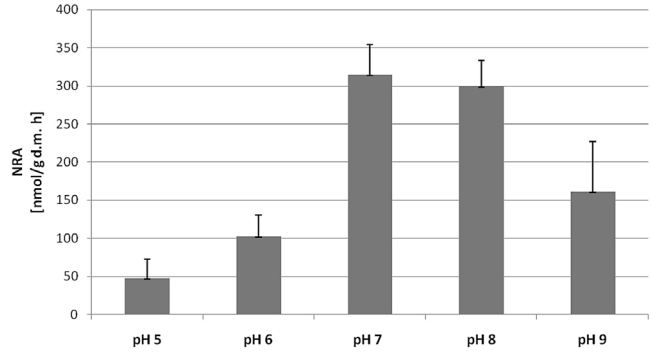

Fig. 4. Nitrate reductase activity is resistant with wide range of pH, but optimum of it placed between pH=7 and pH=8. However differences here were not significant, trend is visible
Rys. 4. Aktywność reduktazy azotanowej utrzymuje się w szerokim zakresie pH, jednakże jej optimum znajduje się w przedziale pH=7 do pH=8. Brak istotności statystycznej różnic

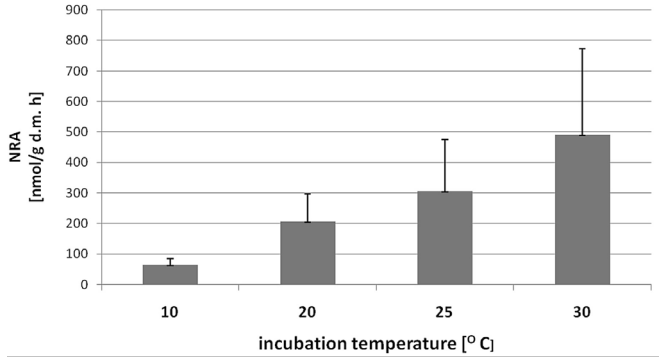

Fig. 5. Temperature have strong impact on nitrate reductase activity. It is statistically significant (rs=0.79, p=0.00002) in spite of high result dispersion in the highest investigated temperature (30 °C)
Rys. 5. Wykazano silny wpływ temperatury na aktywność reduktazy azotanowej. Korelacja ta jest statystycznie istotna (rs=0.79, p=0.00002), pomimo wysokiej dyspersji wyników w najwyższej badanej temperaturze (30 °C)

CONCLUSIONS

1. This research demonstrated that nitrate reductase activity measured in the field conditions may be useful tool for study conditions of plants.
2. Nitrate reductase demonstrate resistance for changing pH of incubation solution.
3. Nitrate reductase activity is strictly depend 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.

POMIAR AKTYWNOŚCI REDUKTAZY AZOTANOWEJ W WARUNKACH TERENOWYCH – METODYKA

Streszczenie

W ciągu ostatnich trzech dekad zainteresowanie biomonitoringiem i badaniami ekologicznymi szybko wzrastało. Dlatego zaistniała konieczność rozwoju nowych metod analiz parametrów biochemicznych, które pozwoliłyby określić biologiczna odpowiedź badanych organizmów na działanie czynników środowiskowych. Głównym celem artykułu jest przedstawienie optymalnych warunków dla analiz kinetyki reakcji enzymatycznych przeprowadzonych w warunkach terenowych.

Aktywność reduktazy azotanowej jest zwykle badane *in vivo* poprzez pomiar produkcji azotynów w tkankach roślinnych, które zostały poddane infiltracji w buforowanym roztworze azotanów. Metodykę oparto o liczne opracowania badawcze oraz wprowadzone własne modyfikacje. Liście dębu zbierano na badanej powierzchni, natychmiast umieszczano w probówkach z roztworem buforowym (fosforan potasu z dodatkiem 0,6% propanolu-1) i poddawano działaniu podciśnienia 0,33 atm. przez 10 min. Następnie dodano znaną ilość azotanu potasu, po czym próbkę roztworu oznaczano dla określenia zerowego stężenia azotynów. Próbki liści inkubowano przez 2 godziny w 20 °C w ciemności. W ten sposób uzyskano warunki stabilności reakcji. Po zakończeniu inkubacji stężenie zsyntezowanego azotynu określono kolorymetrycznie przy użyciu sulfanidamidu oraz N-(1-naftylo)etylenodwuaminy x 2HCl przy długości fali 540 nm. Próbki liści suszono do uzyskania suchej masy. Poziom aktywności reduktazy azotanowej obliczono jako ilość zsyntezowanego azotynu [nmol] na gram suchej masy na godzinę. Uzyskane wyniki wskazują dynamikę zmian aktywności reduktazy odpowiednio do zmian warunków inkubacji.

Przedstawiono dynamikę zmian aktywności enzymu w zależności od pH buforu i temperatury inkubacji. Zaprezentowano również instalację dla przeprowadzenia procesu infiltracji i inkubacji liści.

Słowa kluczowe: reduktaza azotanowa, kinetyka reakcji, metodyka analiz terenowych, optymalizacja reakcji.