METHOD OF MEASUREMENT OF NITRATE REDUCTASE ACTIVITY IN FIELD CONDITIONS

Marek Krywult¹, Dominika Bielec²

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

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ABSTRACT

For the last three decades the interest in biomonitoring and ecological studies has been rapidly growing. Therefore, it was necessary to develop 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 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 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 vacuum 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 KNO₃, 0.1 M K₂HPO₄ 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)
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$_3$. 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 $r_s = 0.79$, $p = 0.00002$ (Fig. 5). But many the authors suggest to use temperature of incubation between 20 and 25 degrees to compare the results with other studies.
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 the activity strictly depends on incubation solution temperature.

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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).

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