VERSITA DOI: 10.2478/cdem-2013-0018 CHEM DIDACT ECOL METROL. 2013;18(1-2):61-68

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NITRATE REDUCTASE ACTIVITY IN CHOSEN MOSSES: A CASE OF STUDY IN SKALNY COLLIERY WASTE TIP

AKTYWNOŚĆ REDUKTAZY AZOTANOWEJ U WYBRANYCH MCHÓW NA PRZYKŁADZIE HAŁDY POWĘGLOWEJ SKALNY

Abstract: Two species of mosses in relation to nitrogen metabolism were examined. This subject is little known in this group of plant. Investigations of nitrate reductase activity in green tissues of *Brachythecium rutabulum* (Hedw.) Schimp. and *Atrichum undulatum* (Hedw.) P.Beauv. were performed. The study was conducted in two localities: heavy contaminated waste tip Skalny located in Upper Silesia, and Blonia City Park in Bielsko-Biala which place was chosen as a control area. For both species high activity of the enzyme was detected. The nitrate reductase activity varied between 99 to 9093 nmol per g dry mass per hour for *B. rutabulum* and 265 to 5135 nmol per g d.m. per hour of nitrite synthesized for *A. undulatum* respectively on Skalny waste tip. In the control area the results varied between 747 to 1077 for *B. rutabulum* and 171 to 518 nmol per g d.m. per hour of nitrite synthesized for *A. undulatum*, respectively. The differences were statistically significant only between the two species but not between habitats probably due to high dispersion and small amount of replications. The levels of nitrate and nitrite in stream water in both areas were also measured. In the Skalny waste tip there were high and reached 1.66 mg · dm^{-3} of nitrite and 65 mg · dm^{-3} of nitrate, respectively. In the control area these amounts were lower and reach zero level for nitrite and 4.5 mg · dm^{-3} of nitrate, respectively.

Keywords: bryophytes, nitrogen fixation, environmental pollution

Introduction

Since many decades mosses are used as a biomonitors. Due to their longevity, high resistance to pollution and other stresses they are used worldwide for these purposes. Very little is known about nitrogen metabolism in this group. Previous studies on nitrate reductase focused mainly on vascular plants [1-4]. Nitrogen is known as one of the most important factors limiting growth of plants [5, 6]. It is available for vascular plants in the form of nitrate and / or ammonia, which may be absorbed by green tissues from soil or from atmospheric fallout [7]. However the availability of nitrate from soil is believed to be the most important factor controlling nitrate reductase activity, gaseous pollutants and nitrate dissolved in precipitation directly absorbed by plants may also affect the total pool of the

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plant available nitrogen [8-10]. The activity of nitrate reductase, the enzyme responsible for nitrate fixation and reduction may also change in plant green tissues as a result of the absorption of nitrate ions [1, 3]. The reduction of nitrate to nitrite plays a key role in nitrate assimilation by controlling its flux to the pathway [11]. Activity of this enzyme also depends on other factors such us temperature, water condition, light intensity and ultraviolet radiation [12, 13]. Little is known about nitrate reductase activity in mosses in general, and nitrogen economy in nitrogen overloaded ecosystems and in ecosystems where nitrogen is the growth-limiting factor. Therefore the aims of our study were: (1) to find out is in mosses nitrate reductase activity takes place, or they use any and other forms than nitrate as source of nitrogen; (2) to study the effect of nitrogen compound saturation on potential nitrate reductase activity; (3) to compare nitrate reductase activity in two different species of mosses existing in the same environment.

Study area

The characteristic of the study area was depicted by Chmura and Molenda [14] in detail. The colliery waste tip Skalny is located in Laziska Srednie (50° 8'27"N, 18°51'19"E) situated in Upper Silesia region (southern Poland). It is s a hill with a relative height of 90 m (Fig. 1). The waste heap occupies 30 ha of an area and the amount of wastes is estimated to be about 17 million Mg. During the years 1912-1998 the wastes were deposited in this area [15]. Since 60s of the 20th century, the intense development of thermal processes, including burning, was observed. Despite land reclamation practices aimed at the liquidation of burning sites which included, among others, the formation of slopes and biological building (sowing of grasses in 1999), the waste heap is still thermally active [14].

Table 1

Description of chemical parameters of sewage water in colliery waste tip “Skalny”

Variable	Value
pH	7.09
Fluorides [mg/dm^3]	1.3288
Chlorides [mg/dm^3]	2062.8083
Carbonates [mg/dm^3]	823.1099
Sulphures [mg/dm^3]	5894.4315
Phosphates [mg/dm^3]	0.1354
Nitrites [mg/dm^3]	1.66
Nitrates [mg/dm^3]	65.0

At the bottom of the studied object there are some outflows of leachate which are formed due to infiltration of precipitation waters. These waters are polluted and are characterized in Table 1. In the surroundings of artificial “river valleys” of streams flowing from outflows of leachate rush vegetation including mosses grows.

Control plot is situated in the Blonia district of the Bielsko-Biala city, in close neighbourhood of the Cyganski Las landscape - nature protected complex (N 49°46’39’’, E 19°03’30.07’’). In this place examined mosses occur on the soil in the mixed forest community.

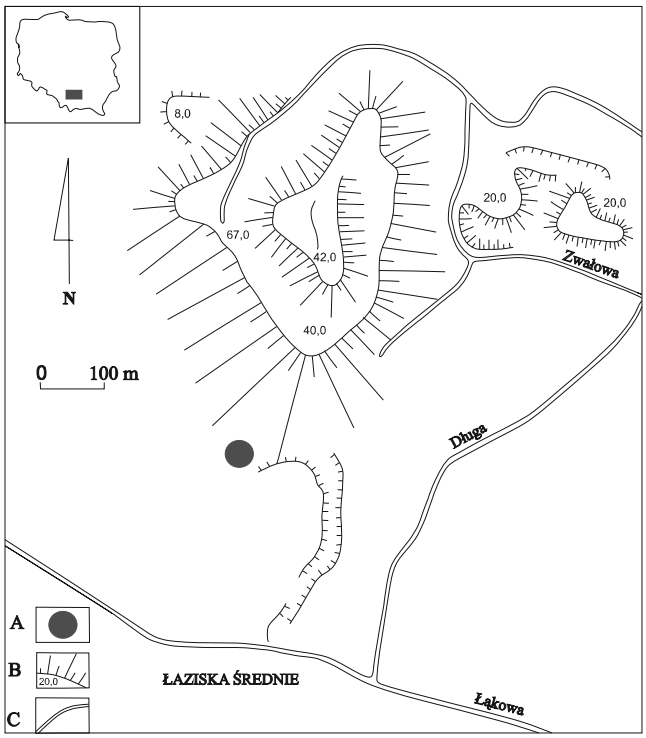

Fig. 1. Localization of the Skalny collier waste tip. Explanation: A - study plot, B - slope of waste tip, C - roads (after [14], modified)

Materials and methods

Investigated mosses

Rough-stalked Feather-moss *Brachythecium rutabulum* (Hedw.) Schimp. - perennial, very wariable robust plant with creeping, prostrate or ascending stem to 15 cm long and erect branches, frequently produces brown, curved, horizontal capsules; spores mature in late autumn to spring. It forms green to golden-green rough mat or wefts on moist basic soil, rocks and at tree bases, on stumps, rotting wood in forests and open semi-shade to partial shade places. Nitrophilous moss abundant on damp, eutrophic woodland sites and grasslands, by rivers and streams and rare or absent on acidic nutrient-poor substrates [16]. It is one of the commonest and abundant pleurocarpous moss in Polish flora from lowland to mountain areas. The species is native to Europe and of temperate region, widespread and frequent throughout the Europe, rare towards the Arctic and in the Mediterranean lowlands. It occurs in Macaronesia, North Africa, Asia, North America, South America, Australia and New Zealand [17].

Common Smoothcap *Atrichum undulatum* (Hedw.) P.Beauv. - perennial, erect, usually robust, to 6-10 cm tall moss, with distinctly transversely undulate narrowly lanceolate, to narrowly lingulate, leaves with 3-6 lamellae. It commonly produces tall red seta and a long cylindrical curved, inclined to horizontal capsules with spores which mature in spring to

early summer. This moss forms yellowish-green to green lax tufts or patches on open humid soils in forests and in damp grassland by rivers and ditches [16]. It is common on moist, moderately acid soils in fertile often shaded sites and is characteristic species for *Fagetalia* order [18], and is abundant in forest communities from *Fagion silvaticae* and *Carpinion betuli* alliances [19]. *Atrichum undulatum* is one of the commonest acrocarpous moss in Polish lowlands and in forest belts in the montane areas. It is circumpolar boreo-temperate moss, frequent or common in deciduous forests throughout Europe, occurs in West, Central and North Asia, North Africa, North and Central America [17].

Measurements of nitrate reductase activity

In the period 03.10-18.10.2012 in three time series nitrate reductase activity was measured in waste tip and one sample was measured in mixed coniferous forest in Bielsko-Biala which was treated as control site. 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 [12, 20, 21] with our own modifications [22-24]. Because in this area no electrical power was supplied, we were used manual vacuum pump our own construction. 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 to avoid an effect of diurnal changes of nitrate reductase activity. The mosses was then subjected to vacuum infiltration (with a manually operated vacuum pump) at 0.33 atm. for 5 minutes and incubated in the buffer solution for 2 hours at 20°C in the darkness. The composition of the incubation buffer was contained by 0.1 M KNO_3, 0.1 M K_2HPO_4 and 0.6% 1-propanol, and adjusted to pH 7.5 using HCL and KOH. Temperature was set up and controlled using hot water or ice cubes due to changes. Construction of the incubation chamber, allow to fast correcting changes of temperature if it was necessary [24]. 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 [20, 25]. Optical density was measured colorimetrically after 10 min at 540 nm using a spectrometer (Hach Lange 5000i, Germany). A mixture of incubation buffer with 1% sulphanilamide in 8% HCl and 0.02% N-(1-naphthyl) ethylene-diamine-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 samples of mosses 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} d.m. h^{-1}).

Nitrate and nitrite concentration in the stream water in Blonia and wastewater flowing out from the waste tip was determined by ion chromatograph (DIONEX 100i, USA).

To examine statistical significance of differences between two bryophyte species and between habitat of colliery waste tip and control was analyzed using t-test and two-way analysis of variance (ANOVA) for interaction. Prior analysis methodological assumptions normal distribution and homogeneity of variance was checked by Shapiro-Wilk and Levene test, respectively.

Results and discussion

The results obtained in these study shows clearly that both of examined moss species demonstrated high activity of the enzyme nitrate reductase (Fig. 2). The nitrate reductase activity varied between 99 to 9093 nmol per g dry mass per hour for *B. rutabulum* and 265 to 5135 nmol per g d.m. per hour of nitrite synthesized for *A. undulatum* respectively on Skalny waste tip. In the control area the results varied between 747 to 1077 for *B. rutabulum* and 171 to 518 nmol per g d.m. per hour of nitrite synthesized for *A. undulatum*, respectively. Differences are only significant between two moss species and are non-significant between habitats (Table 2). The latter result probably is a consequence of high dispersion and small amount of replications because there is distinct trend showing higher activity of the studied enzyme in colliery waste tip (Fig. 2).

Table 2

Univariate two-way ANOVA of the effect of species (*Brachytecium rutabulum* and *Atrichum undulatum*) and habitat on nitrate reductase activity in colliery waste tip and mixed coniferous forest

Effect	Df	Sum Sq	Mean Sq	Probability
Species	1	14793192	14793192	0.04214
Habitat	1	4026613	4026613	0.27602 (ns)
Species · habitat	1	847325	847325	0.61429 (ns)

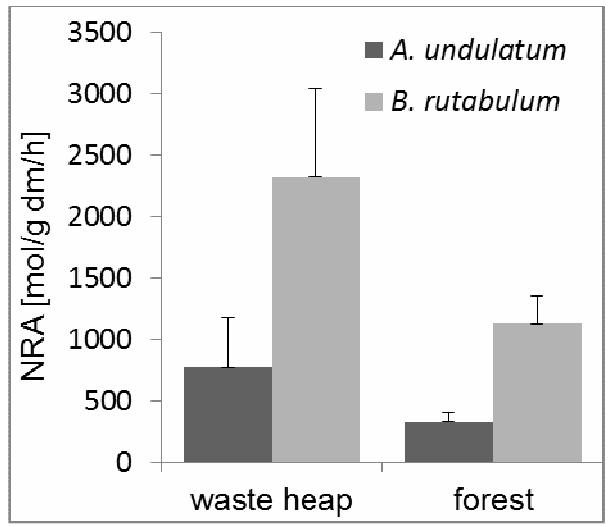

Fig. 2. Comparison of nitrite reductase activity NRA (means±SE) between two moss species in two habitats

The interaction of habitat type and species is also nonsignificant. When species are considered alone difference between them is more significant (t = 4.612, p = 0.039). The NRA is stable because there are no significant differences for NRA combined among time series (F = 1.565, p = 0.226). The activity of nitrate reductase is well investigated in angiosperms [21]. It is a lack of data and knowledge about this metabolic pathway in mosses and pteridophytes. Moreover it was found that *Asplenium africanum* did not

demonstrate activity of nitrate reductase that indicates its use different source of available nitrogen than nitrate [22]. This phenomenon was well described for *Deschampsia antarctica* which may use different sources of available nitrogen forms, both inorganic and organic [26]. Both of investigated mosses demonstrate high activity of enzyme studied. *Brachythecium rutabulum* showed significantly higher activity of nitrate reductase. It may be explained by nitrophilous character of that moss, and the extended enzyme activity in comparison to *A. undulatum* appears to be its physiological adaptation. Measured level of nitrite and nitrate in the waste water from Skalny waste tip was high and reached 1.66 mg dm^{-3} of NO^{-}_{2} and 65 mg dm^{-3} of NO^{-}_{3}, respectively (Table 1). This level of nitrite seems to be toxic for leaving organisms [25]. In the control area these amounts were lower and reach zero level for nitrite and 4.5 mg dm^{-3} of nitrate, respectively. In the wastewater from the waste tip high amounts of chloride and sulfate ions was also observed (Table 1). It may be caused at first by water pumped from coal main to the top of the waste tip, and then by internal processes which take place inside the tip. High resistance of both investigated mosses for extended level of nitrite and nitrate which was found on Skalny waste tip seems to be interesting phenomenon. High tolerance of nitrate reductase for other stress factors such us desiccation, and measured zero levels of nitrate reductase mRNA, which was found for moss *Tortula ruralis* [27] may suggest that mosses nitrate reductase may be significantly different from that found in algae and higher plants. To our best knowledge the present study is the first *in vivo* assay nitrate reductase in terrestrial done in the field conditions. Definitely more research is necessary to explain this very interesting phenomenon.

Conclusions

1. Higher significant nitrate reductase activity in the *Brachythecium rutabulum* then in *Atrichum undulatum* was found for both investigated sites: the colliery waste tip Skalny in Laziska Srednie and Cyganski Las in Bielsko-Biala. These differences may be caused by metabolic adaptation of *Brachythecium rutabulum* to eutrophic environments.
2. Investigated sites do not differ significantly in terms of NR activity, however the trend pinpointing the waste heap as a habitat which induce extended activities of the enzyme for both mosses are visible. Small amount of replications is probably responsible for this situation.

Acknowledgements

Authors thank dr. Dominika Bielec for her help in editing text.

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AKTYWNOŚĆ REDUKTAZY AZOTANOWEJ U WYBRANYCH MCHÓW NA PRZYKŁADZIE HAŁDY POWĘGLOWEJ SKALNY

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Abstrakt: Dwa gatunki mchów badano pod kątem asymilacji azotu. To zagadnienie jest mało poznane u tej grupy roślin. Przeprowadzono badania aktywności reduktazy azotanowej w zielonych tkankach *Brachythecium rutabulum* (Hedw.) Schimp. i *Atrichum undulatum* (Hedw.) P.Beauv. Badania zostały wykonane w dwóch miejscach: na hałdzie powęglowej Skalny na Górnym Śląsku i w parku w dzielnicy Błonia w Bielsku-Białej, które zostało wybrane jako miejsce kontrolne. Dla obu gatunków stwierdzono wysoką aktywność enzymu. Aktywność reduktazy azotanowej wahała się od 99 do 9093 nmol na g suchej masy na godzinę dla *B. rutabulum* i 265 do 5135 nmol na g suchej masy na godzinę azotynu syntetyzowanego u *A. undulatum* na hałdzie Skalny. W miejscu kontrolnym wyniki wahały się od 747 do 1077 dla *B. rutabulum* i 171 do 518 nmol na g sm/h u *A. undulatum*. Różnice były statystycznie znaczące tylko pomiędzy gatunkami, ale nie pomiędzy typem siedliska prawdopodobnie ze względu na wysoką dyspersję i małą liczbę powtórzeń. Zmierzono także poziom azotanów i azotynów w strumieniu wody w obu miejscach. Na hałdzie Skalny zawartość była wysoka i osiągnęła odpowiednio: 1,66 mg dm^{-3} azotynu oraz 65 mg dm^{-3} azotanu. W miejscu kontrolnym wartości te były niższe i osiągnęły odpowiednio poziom zerowy dla azotynów i 4,5 mg dm^{-3} dla azotanu.

Słowa kluczowe: mszaki, wiązanie azotu, zanieczyszczenie środowiska