European Journal of Soil Biology 54 (2013) 41–47

Contents lists available at SciVerse ScienceDirect

European Journal of Soil Biology

journal homepage: http://www.elsevier.com/locate/ejsobi

Original article

Effect of pH on phenol oxidase activity on decaying *Sphagnum* mosses

Teemu Tahvanainen [a,*], Akira Haraguchi [b]

[a] Department of Biology, Faculty of Science and Forestry, University of Eastern Finland, Joensuu Campus, Yliopistonkatu 7, P.O. Box 111, FIN-80101 Joensuu, Finland
[b] Faculty of Environmental Engineering, University of Kitakyushu, Hibikino 1-1, Wakamatsu, Kitakyushu 808-0135, Japan

ARTICLE INFO

Article history:
Received 29 August 2012
Received in revised form
17 October 2012
Accepted 22 October 2012
Available online 5 November 2012
Handling editor: Christoph Tebbe

Keywords:
Bog
Decomposition
Exoenzyme activity
Fen
L-dihydroxy phenylalanine
L-DOPA
Peat accumulation
Water chemistry

ABSTRACT

Inhibition of phenol oxidase (PO) activity by low pH is a potential key mechanism to limit decomposition in acidic *Sphagnum* mires, but few controlled experiments have been performed. The possible effects of assay pH and of auto-oxidation of the commonly used substrate L-dihydroxy phenylalanine (L-DOPA) have remained unreported. We conducted a series of experiments in order to reveal realistic response of PO activity to the pH gradient, taking into account the possible effects of auto-oxidation and assay pH. We found positive responses to pH with 60–100% increase of PO activity with one pH unit (3.5–4.5). In alkaline solutions, L-DOPA was readily auto-oxidized, which may hamper the use of this reagent with alkaline samples. Adjustment of assay pH resulted in variable responses of PO activity to temperature and sample pH. Combinations of matching sample pH and assay pH consistently showed a positive response of PO activity to pH, indicating that control of assay pH is an essential prerequisite of obtaining realistic results of PO activity. The positive relationship of PO activity and pH indicates that the inhibition of enzymic oxidation of phenolic compounds may contribute to the decay resistance of *Sphagnum* in acidic mire habitats.

1. Introduction

Mires dominated by *Sphagnum* mosses are relatively unproductive ecosystems. The average annual biomass production of *Sphagnum* amounts approximately 250 g m^{-2} in the boreal region [1] and reaches 500 g m^{-2} in the warm-temperate zone [2]. However, due to environmental constraints of decomposition caused by low oxygen availability, low pH, poor nutrient availability and low temperatures, globally significant accumulation of carbon takes place in these ecosystems. Peat accumulation is promoted by decay-resistant biochemical traits of *Sphagnum* in interaction with the environmental constraints. In acidic conditions, decay of *Sphagnum* is inhibited by the specific composition of cell-wall polysaccharides [3]. Stalheim et al. [4] showed that the pectin-like polymer sphagnan, isolated from *Sphagnum* cell walls [5], inhibits the growth of certain bacteria, but only by lowering pH. The genus-specific sphagnum acid (p-Hydroxy-beta-[carboxymethyl]-cinnamic acid) [6] and other phenolic compounds [7] are suggested to contribute to the decay resistance of *Sphagnum* by forming an amorphous protective layer on cell-wall surfaces that acts as a physicochemical barrier for micro-organisms [8]. However, the exact role of Sphagnum phenolics remains debatable [9,3]. Phenolic compounds are oxidized by extracellular phenol oxidase enzymes (PO from here on) that are constrained by low oxygen availability in water-saturated peat [10]. In aerobic conditions, where most of the decomposition takes place, low nitrogen concentration [11] and acidity [12,13] are suggested to limit the oxidation of phenolics.

Sphagnum mosses are dominant in bogs and poor fens that are acidic environments with typical water pH range 3.5–4.5 [14]. In neutral to alkaline pH, *Sphagnum* growth becomes weaker [15] and the rate of net photosynthesis declines [16]. It is unclear, however, what causes the relative scarcity of *Sphagnum* and slows down *Sphagnum* peat accumulation in moderately acidic fens (pH 4.5–6.5). One possible mechanism is the failure of the biochemical resistance to decay of *Sphagnum*, as indicated by the positive relationship between pH and decomposition of bleached, phenolic-free *Sphagnum* holocellulose [3]. Oxidation of phenolic compounds should also respond positively to pH, since phenolic compounds are more readily oxidized when dissociated. Indeed, increase of phenolic oxidation with increased pH has been observed in *Sphagnum* peat samples in some studies [12,13], although these studies did not differentiate between enzymic oxidation and potential auto-oxidation at the near-neutral to alkaline pH levels

* Corresponding author. Tel.: +358 504423987; fax: +358 13318039.
E-mail addresses: teemu.tahvanainen@uef.fi (T. Tahvanainen), akhgc@kitakyu-u.ac.jp (A. Haraguchi).

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http://dx.doi.org/10.1016/j.ejsobi.2012.10.005

where the response was observed. Toberman et al. [17] found a positive correlation of pH and PO activity in peat, in connection to the acidifying effect of drainage, while Straková et al. [18] failed to find this correlation in litter samples from the same sites. Fenner et al. [19] connected the increase of PO activity to elevated pH in a rewetted peatland. It appears that controlled experiments on the effects of pH to enzymic degradation of *Sphagnum* phenolics are missing, however.

We conducted a series of experiments in attempt to unravel the effects of pH on PO activity on decaying *Sphagnum* shoots. We focused our enzyme assays on segments of *Sphagnum* shoots below the pigmented, living part, i.e. on the early phase of decomposition. We used the phenolic substrate L-DOPA (L-dihydroxy phenylalanine), which is the most commonly applied method in PO assays of peat. Earlier studies with L-DOPA have not considered the potential effects of differences of pH between the natural or experimental conditions and the enzyme assay. Also the role of auto-oxidation of the substrate in response to pH is unclear, although it is known to be a problem in the medical use of the chemical [20]. We controlled pH of the enzyme substrates, in order to reveal potential auto-oxidation and effects of change of pH during the assay on enzyme activities. We aim to answer the questions: 1) Does the phenolic substrate L-DOPA show auto-oxidation that could affect interpretation of pH response of PO activity? 2) Does the manipulation of assay pH affect the results of response of PO activity to experimental pH or temperature? 3) We also aim at the estimation of realistic responses of PO activities to the pH gradient. We repeated our experiments with variation of experimental setup and using several *Sphagnum* species.

2. Materials and methods

2.1. Sample preparation and enzyme assays

We collected monospecific samples of *Sphagnum* mosses from Kiimasuo, near the city of Joensuu, in Eastern Finland (62° 36′, 29° 31′), and from the Bougatsuru mire (33° 05′, 131° 15′) and the Tadewara mire (33° 07′, 131° 14′) in Kyushu, South-western Japan. The Finnish site is located at 85 m ASL in the middle-boreal zone, with mean annual temperature +2 °C and precipitation 660 mm^{-yr}. The Finnish site represented typical ombrotrophic bog vegetation characterized by dominance of *Sphagnum fuscum* (Schimp.) H. Klinggr., *Eriophorum vaginatum* L. and dwarf shrubs. The Japanese sites are located in the warm-temperate zone at the elevations 1200 m (Bougatsuru) and 1000 m (Tadewara), with mean annual temperature +8 °C and precipitation 2700 mm^{-yr}. The Japanese sites are characterized by high biomass, especially of *Phragmites australis* (Cav.) Trin. ex Steudel and *Moliniopsis japonica* (Hack.) Hayata. Only two *Sphagnum* species, *Sphagnum fimbriatum* Wilson and *Sphagnum palustre* L., are found in limited zones of these mires.

We measured activities of phenol oxidase (PO) from *Sphagnum* shoot segments below the living, normally pigmented apical parts. All reported enzyme assays, thus, focus on the early phase of decomposition of fresh plant material, the dying *Sphagnum*. The exact dimensions and mass of the segments were varied according to differences between species. All enzyme assays were conducted in 10 ml glass vials. The *Sphagnum* shoot segments were placed in the vials and total volumes of 3–5 ml of reagent solutions were added. The volume was adjusted according to the size of samples of different species, resulting in the approximate ratio of 2.5 mg dry weight per 1 ml of substrate solution. In all assays, the vials were regularly shaken and incubations were halted by placing the vials in ice bath. Immediately after cooling, the samples were filtered through laboratory-grade tissue paper to second vials in the ice bath. This treatment was sufficient since the solutions were clear and we only needed to exclude fragments of *Sphagnum*. The *Sphagnum* shoot segments were dried at +70 °C to constant weight and dry weight was determined with 0.1 mg accuracy. In all assays, we used enzyme substrates adjusted to different pH levels by pH buffers (phthalate buffer pH 4.01, phosphate buffer pH 7.00) and additions of HCl and NaOH.

The PO assay was based on measuring the absorbance (460 nm) caused by the oxidation of 5 mM L-dihydroxy phenylalanine (L-DOPA) into 2,3-dihydroindole-5,6-quinone-2-carboxolate (DIQC) with 9 min reaction time (Pind et al., 1994). The PO activity was calculated as nmol g^{-1} min^{-1} DIQC produced using the equation:

$$\mathrm{DIQC}\left(\mathrm{nmol\ g^{-1}\ min^{-1}}\right) = abs/\left(3.7\times10^{-5}\right) \times v\ (\mathrm{l})/m\ (\mathrm{g})/t\ (\mathrm{min}) \quad (1)$$

where v is the total volume (l) of the enzyme assay, m is the dry weight (g) of the *Sphagnum* sample and t is the incubation time (min) with L-DOPA. The absorbance coefficient 3.7×10^{-5} $nmol^{-1}$ DIQC is used following Pind et al. [12]. The increase of absorbance (abs) was calculated as difference of absorbance of the L-DOPA-incubated sample (abs_{sample}) and absorbance of blank sample incubated with Milli-Q water (abs_{blank}) and the L-DOPA reagent-blank without a sample ($abs_{reagent}$) using the equation:

$$abs = abs_{sample} - abs_{blank} - abs_{reagent} \quad (2)$$

where all absorbance values were subtracted for absorbance of Milli-Q water. With the modification of subtracting $abs_{reagent}$ we wanted to take into account the possible auto-oxidation of L-DOPA during the assay.

Spectrophotometric measurements were conducted using 96-well microplates with a Perkin Elmer 1420 Multilabel Counter.

2.2. Experiment 1 – effect of pH on auto-oxidation of L-DOPA

We conducted a small experiment to demonstrate the effect of pH on the auto-oxidative development of DIQC, at room temperature (24 °C). A six-step pH series was prepared and these pH-adjusted solutions were mixed with 10 mM L-DOPA in proportion corresponding to our enzyme assays (1:2). Absorbance measurements were conducted after 9, 20, 40 and 120 min and concentration of DIQC (μM) was calculated after subtracting the corresponding reagent absorbance from the absorbance of the pH-adjusted 5 mM L-DOPA solutions (abs_{sample}) using the equation:

$$abs(\mathrm{t}) = abs_{sample} - \left(abs_{pH} + abs_{DOPA}\right)/2 \quad (3)$$

where abs_{pH} is the absorbance of pH-adjusted solution and abs_{DOPA} is absorbance of unadjusted 10 mM L-DOPA. The pH values of the 5 mM L-DOPA series were 3.36, 3.96, 5.05, 6.91, 7.74 and 8.29.

2.3. Experiment 2 – effects of temperature and assay pH

Samples of *S. fuscum* and *S. palustre* were used in this experiment, where we explored the temperature acclimation of PO activity, subject to the manipulation of assay pH. The *S. fuscum* sample was collected from the Finnish site (see Section 2.1) 29th September 2011. Water pH was 4.2, EC was 21 μS cm^{-1} and temperature of peat at the water level was +10 °C at the sampling site. The *S. palustre* sample was collected from the Bougatsuru mire 6th October 2011. Water pH was 4.3, EC was 67 μS cm^{-1} and temperature of peat at the water level was +14 °C at this site. Both samples were acclimated in sealed plastic bags in the dark at 10 °C for two weeks before the analysis. The PO assays were performed at